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AGGLUTINATION REACTIONS IN RHEUMATOID ARTHRITIS*

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BACTERIOLOGIC investigations in rheumatoid arthritis have been undertaken frequently since Billings, Rosenow, and others, advanced the theory of focal infection as a possible etiologic agent in this disease.

A considerable stimulus to this type of investigation was given by the work of Cecil, Nicholls, and Stainsby,[1] who reported the isolation of a streptococcus from the blood stream of 61.5 per cent of 78 patients with rheumatoid arthritis —83.3 per cent of the positive cultures yielded organisms culturally and biologically identical—the "typical strain"—an attenuated hemolytic streptococcus.

Although others were unable to confirm this finding of positive blood cultures in rheumatoid arthritis, attention was turned to the fact that these "typical strains" of hemolytic streptococci were found by Nicholls and Stainsby[2] to be agglutinated in high dilutions by the sera of a large percentage of cases of this disease; 103 cases in 110 (93 per cent) had agglutinations of 1:640 or higher. Among 218 controls the majority were negative, and when agglutination was present it usually was in titer of less than 1:160. Hemolytic streptococci from other sources gave comparable reactions in lower titer. *Streptococcus viridans* gave weakly positive reactions. Sera from patients with other types of arthritis, febrile and nonfebrile diseases, and normal people, in most instances failed to agglutinate the organisms in significant titer.

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This work was repeated by Dawson, Olmstead, and Boots[3-5] who found no correlations between the source of the hemolytic streptococci and the agglutination titers obtained. Absorption tests failed to show specificity of the agglutination reaction for the strains of hemolytic streptococci examined. They found that in 206 patients with rheumatoid arthritis, 113 (54.8 per cent) had agglutinations of 1:160 or higher. A total of 256 control cases were negative or the agglutinations were of lower titer. They felt that there was a definite relation between the agglutination reaction and the age of the patient, and the duration of the disease. Examination of 41 other strains of gram-positive cocci failed to show comparable titers of agglutinations except tests using rough strains of pneumococci.

Blair and Hallman[6] examined the sera of 62 patients with rheumatoid arthritis and found that 82 per cent of them agglutinated "typical strain" AB66 in dilution of 1:160 or higher. Strains of hemolytic streptococci from other sources were agglutinated less frequently or in lower titers. A total of 32.9 per cent of 94 controls were positive in dilution of 1:160 or higher. They were unable to demonstrate any correlations between the agglutination titer and the age of the patient, the duration of the disease, the number of joints involved, or the sedimentation rate.

Gray and Gowen[7] found agglutination in some degree in 52 of 60 cases (86.6 per cent) against one of the "typical strains," AB13-45 (75 per cent) were in a dilution 1:160 or higher. The majority of cases of long standing showed agglutination in low titer, if at all.

Dawson and Wetherby[8] reported a strain of hemolytic streptococci isolated from a patient with chronic arthritis, agglutinated by 98.1 per cent of 60 persons with chronic arthritis and 28 per cent of controls in a titer of 1:400 or higher.

Cox and Hill[9] found that 197 sera from patients with rheumatoid arthritis agglutinated "typical strain" AB13 in approximately 70 per cent of cases in a titer of 1:320 or higher. Other organisms, including another strain of *Streptococcus hemolyticus*, *Streptococcus viridans*, *Streptococcus anhemolyticus*, *Staphylococcus aureus*, and *B. coli* were agglutinated in dilutions of 1:320 or higher in from 8 per cent to 49 per cent of cases.

McEwen, Bunim, and Alexander,[10] using a scarlet fever strain NY5, report positive agglutinations in 86 per cent of 36 patients with atrophic arthritis, 56 per cent in a dilution of 1:160 or higher. None of 35 normals had positive agglutinations.

Goldie and Griffiths[11] found that 80 per cent of 31 patients with rheumatic fever and chorea, 89 per cent of 28 patients with chronic infectious arthritis, 71 per cent of 17 patients with "streptococcal diseases," and 10 per cent of controls agglutinated stock strains of hemolytic streptococci in a dilution of 1:160 or higher.

Hartung, Davis, Steinbrocker, and Straub[12] report "typical strain" AB13 agglutinated by 36 per cent of 50 patients with rheumatoid arthritis 1:160 or higher. Two strains of hemolytic streptococci from other sources were agglutinated in 30 per cent and 24 per cent by the same sera. Ten per cent of normals were positive.

Tillett and Abernathy[13] found that in a number of bacterial infections other than streptococcal, the sera of the patients are capable of agglutinating certain strains of hemolytic streptococci. The reaction is demonstrable soon after the onset, persists during the active stage, and disappears after recovery. This agglutinability is striking when live organisms or organisms killed by formalin are used, but disappears if cultures are heated to the thermal death point.

Keefer, Myers, and Oppel,[14] therefore, performed a large group of agglutination tests using heat-killed organisms. The sera of 12 patients in 22 (55 per cent) with rheumatoid arthritis gave positive reactions; 9 patients (41 per cent) had agglutinations in a titer of 1:160 or higher. Only one serum agglutinated in a titer above 1:640. Positive reactions were obtained in 26.2 per cent of patients with rheumatic fever (42), 9.7 per cent of rheumatic heart disease,[31] 14.3 per cent of degenerative arthritis,[28] 23.5 per cent of erysipelas.[17] Sera from patients with other forms of arthritis, and a number of infectious and noninfectious diseases failed to agglutinate heat-killed streptococci. The reaction in patients with rheumatoid arthritis persisted for one to several months. In some cases it was maintained by intravenous vaccine.

Wainwright[15] performed a series of agglutinations with the sera of 51 patients with rheumatoid arthritis using both heat-killed and living hemolytic streptococci. He found the heat-killed organisms were agglutinated in slightly lower titers, but otherwise the curves were similar.

Wainwright,[16] in another investigation, found that agglutination of hemolytic streptococci by sera of patients with rheumatoid arthritis was not confined to organisms of group A (Lancefield), or to streptococci from human sources. Absorption with a representative strain of one group removed the agglutinins not only for that strain but for others of the same group—bearing out group specificity of the reaction rather than strain specificity.

McEwen, Chasis, and Alexander[17] did similar agglutination tests using strains of hemolytic streptococci of Lancefield's groups A, B, C, D, E, F, and G. The strongest agglutinations occurred in group A, but definite reactions were also obtained in the other groups. Crude C extracts of all groups were prepared, and when these were tested with the sera of patients with rheumatoid arthritis, occasional crossing was found with the extracts of all groups, and frequently with those of groups A, B, and G.

Chasis and McEwen[18] pursued this line of investigation further and found that cross reactions occurred with crude C extracts of hemolytic streptococci of the various groups, when used in precipitin tests with immune human sera and with immune rabbit sera, and the sera of patients with rheumatoid arthritis, rheumatic fever, and convalescent scarlet fever. This reaction was thought to be due to the presence of antibodies in the sera against a nongroup specific ration present in the bacteria and in the crude C extracts, this substance being of nonprotein nature.

Dawson and Olmstead[19] examined the sera of 76 patients with rheumatoid arthritis for agglutinins against organisms in Lancefield groups A, B, C, D, E, F, and G. Using group A they found the percentage of positive reactions to be approximately the same as they had reported previously,[4] or about 55 per cent.

However, there were only three instances of definite positive reactions (1:160 or above) with organisms other than group A, and two of these were strongly positive for group A also. Control sera agglutinated groups B to G in as high dilution as did the sera of patients with rheumatoid arthritis. Control sera which agglutinated group A organisms in titers comparable with that of rheumatoid arthritis were found only in proved severe hemolytic streptococci infections.

Dawson, Olmstead, and Jost[20] compared the agglutinins and precipitins against hemolytic streptococci in 71 patients with rheumatoid arthritis and in 79 controls. They demonstrated a close approximation but not an absolute agreement between the capacity of rheumatoid arthritis sera to agglutinate strains of hemolytic streptococci and to precipitate various group-specific fractions of this organism. Bunim and McEwen[21] found no correlations between the antistreptolysin titer and the hemolytic agglutination titer. Eighty-five per cent of 205 sera from 75 patients with atrophic arthritis had antistreptolysin titers within normal limits regardless of the stage of the disease or severity of clinical symptoms.

Short, Dienes, and Bauer[22] compared the results obtained in 49 patients with typical rheumatoid arthritis, using the sedimentation rate, Arneth-Schilling counts, the Vernes resorcinol test, and agglutination tests with hemolytic streptococci. A total of 92.2 per cent of the patients showed elevated sedimentation rates; 87 per cent of the Schilling counts were positive (over 8 per cent young cells), 58.3 per cent of the Vernes tests were positive; 53.1 per cent of agglutinations showed a titer above 1:160 for at least one of the four strains employed.

The evidence which has accumulated from these investigations shows that in rheumatoid arthritis there are, in a fairly large percentage of cases, positive agglutinations for hemolytic streptococci, regardless of the source of the organisms. This reaction comes on during the course of the disease, persists a variable length of time, varying in strength, and usually is low in titer or absent when the disease reaches a quiescent stage. Heat-killed streptococci are agglutinated similarly but in lower titer than with live organisms. Agglutinations are strongest with organisms of Lancefield's group A, but are present in other groups, and definite cross reactions frequently occur. There seems to be a close relationship between the agglutinins and precipitins for hemolytic streptococci, but none between agglutinins and antistreptolysins in rheumatoid arthritis. Elevated sedimentation rates and positive Schilling counts are considerably more constantly present than the resorcinol test and the agglutination reaction.

As indicated in the preceding reports, there is a wide variation in the results of the agglutination tests in different hands, and marked differences of opinion as to their significance.

Consequently, we performed a series of agglutination tests on patients with typical rheumatoid arthritis, with osteo-arthritis, with a number of related conditions, and on normal controls using a number of strains of hemolytic streptococci from various sources, and with two strains of *Streptococcus viridans*. This investigation was undertaken in an attempt to evaluate the agglutination reaction in relation to the patients seen in out-patient arthritis clinic.

METHOD

The medium used for cultures was dextrose broth. Flasks of this media were inoculated with a diffuse fairly dense culture and incubated at 37° C. for fifteen to twenty-four hours.

Serum dilutions were made with 0.8 per cent sodium chloride solution in proportions of 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1,280, 1:2,560, and 1:5,120. To these dilutions 0.5 c.c. of culture was added, making the final dilutions 1:20, 1:40, 1:80, 1:160, 1:320; 1:640, 1:1,280, 1:2,560, and 1:5,120. A control consisting of 0.5 c.c. of culture and 0.5 c.c. of broth was included in all the tests.

TABLE I

AGGLUTINATION TITER OF SERA OF PATIENTS WITH ARTHRITIS WITH HEMOLYTIC STREPTOCOCCI

TYPE OF CASE	TITER OF AGGLUTINATION											PERCENTAGE POSITIVE	PERCENTAGE NEGATIVE
	NEGATIVE	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	TOTAL CASES		
Typical rheumatoid arthritis	3	1	3	8	5	5	4	4	1	2	36	58	42
Typical osteoarthritis	8	1	4	5	4	2	0	0	1	0	25	28	72
Related diseases	5	0	1	1	1	0	1	0	1	0	10	30	70
Controls	19	1	8	3	0	2	1	1	1	0	36	13.9	86.1

Agglutination tests were placed in a water bath at 56° C. for two hours and then overnight in the icebox. The tests were read at the end of two hours and after overnight refrigeration.

Readings were made after gently tapping the tubes until the bacteria had risen from the bottom. The reactions were recorded by the following scale:

++++ Disk or firm clump—broth clear.
+++ Loose clumps, readily broken—broth clear.
++ Small clumps—broth clear.
+ Small but distinct clumps—broth cloudy.
- No clumps visible.

The highest dilution in which distinct and small clumps were observed was considered the end point or titer. The hemolytic activity of the strains employed was tested on blood agar.

Six strains of hemolytic streptococci were used in the agglutination tests. They were obtained from the following sources:

Strain I. From the throat of patient with scarlet fever.

Strain II. From patient with erysipelas.

Strain III. From throat of patient with rheumatoid arthritis and nephritis. This strain produces typical rheumatoid arthritis in rabbits.

Strain IV. From throat of patient with nephritis.

Strain V. From throat of patient with nephritis.

Strain VI. From dog tonsil.

Agglutination test was performed with at least three of these strains with the serum of each patient.

A smaller number of agglutination tests were performed with two strains of green streptococci obtained from the following sources:

Strain 1. From extracted tooth of patient with rheumatoid arthritis.
Strain 2. Blood culture from patient with subacute bacterial endocarditis.

The results are summarized in Table I. Agglutination titers of 1:160 or higher were obtained in 21, or 58 per cent, of 36 patients with typical rheumatoid arthritis. In 25 patients with typical osteo-arthritis, agglutination titers of 160 or higher were obtained in 7, or 28 per cent; in 10 patients with related diseases—rheumatic fever, fibrositis, and gonorrheal arthritis—positive agglutination reactions were obtained in 3, or 30 per cent. In the control group of 36 patients positive agglutination reactions were obtained in 5, or 13.9 per cent.

TABLE II

RELATIONSHIP BETWEEN DURATION OF DISEASE AND AGGLUTINATION REACTION IN RHEUMATOID ARTHRITIS

DURATION OF DISEASE	NEGATIVE	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2,560	1:5120	TOTAL CASES
Six months or less				2	2	1	1				6
One year		1		1	1					1	4
One to five years			3	5	2	3		2			15
Over five years	4			1	2	1	2			1	11
Total											36

DURATION

One Year or Less	Over One Year
Positive—6 cases, or 60 per cent 1:160 or higher	Positive—13 cases, or 50 per cent
Negative—4 cases, or 40 per cent under 1:160	Negative—13 cases, or 50 per cent

TABLE III

AGGLUTINATION TITER OF SERA OF PATIENTS WITH ARTHRITIS WITH STREPTOCOCCUS VIRIDANS

TYPE OF CASE	TITER OF AGGLUTINATION										TOTAL CASES	PERCENTAGE POSITIVE
	NEGATIVE	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120		
Typical rheumatoid arthritis	9		4	3	3		1				20	20
Typical osteo-arthritis	4		3	1	1	1					10	20
Related diseases												
Controls	7	1	1	1		1					11	9

The relation of the duration of the disease to the agglutination titer is shown in Table II. In this series there seemed to be no relationship between the two conditions. The results obtained with the two strains of *Streptococcus viridans* are summarized in Table III. Agglutination titers of 1:160 or higher were obtained in 4, or 20 per cent, of 20 patients with rheumatoid arthritis, with 2, or 20 per cent, of 10 patients with osteo-arthritis and in 1, or 9 per cent, of 11 controls.

SUMMARY AND CONCLUSIONS

Previous experimental work on this subject is reviewed briefly. The agglutination titer of sera of 36 patients with rheumatoid arthritis, of 25 patients with osteo-arthritis, of 10 patients with related conditions, of 36 normal controls with strains of hemolytic streptococci obtained from various sources was determined. Agglutination titers of 1:160 or higher were obtained in 58 per cent of the patients with rheumatoid arthritis; in 28 per cent of those with osteo-arthritis, in 30 per cent of those with related conditions and in 13.9 per cent of the controls.

In this series there seemed to be no relationship between the duration of the disease and the agglutination titer.

Agglutination titers of 1:160 or higher were obtained with two strains of *Streptococcus viridans* in 20 per cent of 20 patients with rheumatoid arthritis, in 20 per cent of 10 patients with osteo-arthritis and in 9 per cent of 11 controls.

Although the percentage of positive agglutination reactions is considerably higher in patients with rheumatoid arthritis than in patients with osteo-arthritis, related conditions and controls, the reaction is of little value as a diagnostic measure, because patients with typical rheumatoid arthritis may have negative tests, and controls give positive reactions often enough to be confusing. The test is time-consuming, tedious, and difficult to perform accurately. No positive information is derived from agglutination reactions as to the etiology of the disease.

In the present state of immunological knowledge the significance of the agglutination reaction in rheumatoid arthritis is largely a matter of conjecture and is nothing more than suggestive. Whether infection by the hemolytic streptococcus plays a role of primary or secondary importance in the production of the disease, will require more evidence than that obtained from agglutination reactions.

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INTRAMUSCULAR ADMINISTRATION OF SODIUM SULFAPYRIDINE TO CHILDREN*

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THOUGH a report[1] on the intramuscular administration of sodium sulfapyridine to patients appeared as early as July, 1939, this route has not been employed extensively. A recent favorable report[2] in this JOURNAL on the intramuscular route for parenteral administration of sodium sulfapyridine prompted the publication of our clinical experience and experimental work with the intramuscular administration of this drug.

Because such injections usually must be given by a physician, it seemed desirable to give them as infrequently as practicable. Our experience leads us to believe that in most infants and young children a satisfactory blood sulfapyridine level can be maintained by an intramuscular injection of sodium sulfapyridine no oftener than every twelve hours.

One hundred and ten intramuscular injections of a 30 per cent aqueous solution of sodium sulfapyridine monohydrate were given to 71 patients. The

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dosages, calculated as sodium sulfapyridine monohydrate, were approximately as follows:

16 injections of 0.05 Gm. per kilogram of body weight
75 injections of 0.1 Gm. per kilogram of body weight
18 injections of 0.2 Gm. per kilogram of body weight
1 injection of 0.3 Gm. per kilogram of body weight

When the total volume to be given amounted to more than 10 c.c., the dose was divided and given into two sites. Most of the injections were given as an initial dose to patients who were critically ill with pneumonia, and usually oral administration of sulfapyridine was begun within a few hours. The injections were given deep into the gluteal or quadriceps muscles. The number of injections in each patient was as follows:

53 patients received 1 injection
9 patients received 2 injections
4 patients received 3 injections
1 patient received 4 injections
2 patients received 5 injections
1 patient received 6 injections
1 patient received 7 injections

For 21 patients oral administration of sulfapyridine was withheld for one to twenty-four hours in order to determine the course of the blood sulfapyridine level after a single intramuscular dose (Table I).

TABLE I

BLOOD SULFAPYRIDINE LEVELS FOLLOWING A SINGLE INTRAMUSCULAR DOSE OF A 30 PER CENT AQUEOUS SOLUTION OF SODIUM SULFAPYRIDINE MONOHYDRATE

The dosage is expressed as grams of sodium sulfapyridine monohydrate per kilogram of body weight.

CASE	WEIGHT KG.	DOSE GM./KG.	FREE SULFAPYRIDINE LEVELS IN MG./100 C.C. OF BLOOD				
			HOURS AFTER INJECTION				
			1	4	6	12	24
1	18	0.06	3			3	
2	22	0.06	2			3	
3	6.7	0.1	8				
4	6.8	0.1	3			4	
5	7.2	0.1	4		8		
6	8.5	0.1	5				
7	11	0.1	3	5			
8	12	0.1	5		8		
9	15	0.1				7	
10	16	0.1	7				
11	16	0.1	5		7	5	0.8
12	18	0.1	4		8	5	
13	19	0.1	3		8	5	0.9
14	22	0.1	4				
15	25	0.1	5				
16	9.4	0.2	6		11	8	3
17	15	0.2				12	
18	18	0.2	6		10	6	4
19	19	0.2	9	13	12	9	2
20	22	0.2	9	12	11		5
21	13	0.3	8			15	

The local reaction in most cases was surprisingly slight. Considerable pain was a complaint during the injections, but it subsided within a few minutes. Slight tenderness and, in a few cases, some induration at the site of injection persisted for a day or two. Due to a misunderstanding one injection was given subcutaneously, resulting in a slough about 3 cm. in diameter. The patient had a staphylococcus bacteriemia, which resulted in death a few days later. In one fat infant a deep slough about 3 cm. in diameter followed an injection of 0.2 Gm. per kilogram of body weight (6 c.c.) into the gluteal region. Since the depth of the subcutaneous fat in this infant was nearly as great as the length of the needle used, it is possible that the solution was not given into the muscle. In another infant the skin at the site of the needle puncture became necrotic for an area of about 1 cm. in diameter; this healed without ulceration.

If one uses the intramuscular route sloughs from accidental subcutaneous administration must be expected occasionally, but with proper precautions they should rarely occur. It is important to remember that on a well-nourished infant the subcutaneous fat in the gluteal region may be more than an inch thick.

From two patients who died one day after injection, specimens of muscle were obtained from the injected sites. The only gross changes noted were hemorrhage into the muscle in both cases and the deposition of a few small crystals in one. Microscopically there were hemorrhage, infiltration with polymorphonuclear neutrophiles, and swelling and loss of cross striation of the muscle fibers.

In order to obtain further information regarding the severity of the damage to the muscle, each of ten rabbits was injected with 5 c.c. of the solution into the extensor muscles of the thigh. Although this volume amounted to a very large dosage for the rabbits, it was chosen because it represented about the average volume given to patients. The rabbits were autopsied at intervals of one to thirty-three days after the injections. The reaction in the muscle in all the rabbits was limited in the main to an area about 3 cm. in diameter. In some rabbits there were changes along the fascial planes for a slightly greater distance.

Rabbits autopsied on the first, second, third, and fifth days showed similar changes, except that crystals were found in the muscle only in the first rabbit. The crystals extended throughout an area about 1 cm. in diameter. Hemorrhage was the only other gross change noted. Microscopically, there were hemorrhage, edema, infiltration with polymorphonuclear neutrophiles, and round cells, and loss of the staining properties of the muscle fibers.

The gross change in the muscles of the rabbits autopsied on the ninth, thirteenth, eighteenth, and twenty-third days consisted only of a brownish discoloration. Microscopically, there were degenerative changes in the muscle fibers; infiltration with polymorphonuclear neutrophiles, round cells, and giant cells; and some increase in fibrous tissue.

The muscles from the two rabbits autopsied on the thirty-third day showed no gross changes, but microscopically, there was an infiltration with giant cells and an increase in fibrous tissue.

CONCLUSION

A 30 per cent aqueous solution of sodium sulfapyridine monohydrate may be given intramuscularly to children when other routes of administration are not practicable. Following a dose of 0.1 Gm. per kilogram of body weight, a blood sulfapyridine level between 5 and 10 mg. per cent may be expected throughout most of the first twelve hours. The danger of a slough should not be great if proper precautions are taken to insure injection into the muscle. Although crystals (presumably sulfapyridine) may precipitate at the site of the injection, blood level studies on patients suggest that most of the solution is absorbed during the first twelve hours, and autopsy studies on rabbits demonstrate that crystals do not remain long at the site of the injection. The intramuscular injection of sodium sulfapyridine results in prolonged and perhaps permanent damage to the muscle, but the area involved is not large. The pathologic studies suggest that it may be undesirable to give more than one injection into any one site.

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I. DAILY VARIATIONS IN THE TOXICITY OF NEOARSPHENAMINE IN RABBITS*

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IN THE last decade Petersen[1] has shown that the "normal fluctuations" in human blood chemical levels are definitely interrelated and correlated with the weather. He also called attention to the possibility of variations in response of human beings to drugs in connection with this constantly changing physiologic state of the body and on his suggestion experimental investigations with morphine sulfate on mice were performed (Nedzel,[2-4] and Sargent and Nedzel[5]). It was found that the meteorologic conditions definitely varied the toxicity of morphine sulfate. Previously Macht[6] found a very definite influence of barometric pressure and other meteorologic conditions on the potency of digitalis for cats, and pointed out that the difference in the toxicity is due to changes in the physiologic functions of the cats, more particularly to changes produced in the respiration and circulation by the fall in barometric pressure. He concluded that fluctuations in barometric pressure and other changes in atmospheric conditions play an important role in the action of various drugs.

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Here we present the observations on rabbits, which were administered neoarsphenamine. The animals used were of the same breeds, approximately of the same age. The females were all virgins, and in each daily group the same number of male and female animals was used. All animals were kept in an air-conditioned room (controlled temperature) under the same care. The diet consisted of Vitality Rabbit Ration in pellets, lettuce, and carrots. Water was supplied freely.

The experiments were performed in groups. Each day the same number of animals was injected with the same dose of neoarsphenamine per weight. The neoarsphenamine used in all these experiments was of the same lot, dissolved under similar conditions. The solution of the neoarsphenamine was never older than six minutes from the time the ampoule was opened, and was given intravenously in the ear vein. The injections were given at the same time in the morning.

The dose selected was established on the basis of an average death rate of approximately half of the number of the animals injected and was found to be 0.42 Gm. of neoarsphenamine per kilogram weight of the rabbit, as best to serve our purpose.

Winter Group.—One hundred and twenty rabbits were used in the first series, 10 rabbits being injected daily for twelve consecutive days, beginning February 17. The second series of experiments, which was started September 19, contained 150 rabbits, and the procedure was the same, namely, every consecutive day for fifteen days, 10 rabbits were receiving the same dose of neoarsphenamine as in the first group. The date of injections and the date of death, as well as the maximal and minimal daily temperature and barometric pressure for these days, are presented on Chart 1.

In the upper part of the chart are recorded the days on which the rabbits were given the neoarsphenamine intravenously, and are designated by a black dot. Each cross represents the death of one animal and is plotted on the day when this death occurred. The black lines, starting from the black dot, are extended to the right, and the crosses on these lines represent the death of a single animal on a certain day and indicate the group to which the animal belonged. Below this, the wide black field with white line in the center indicates the official temperature. The upper margin gives daily maximum temperature; the lower, the minimum temperature; the middle white line presents the mean temperature.

The lower curve indicates the official barometric pressure. Vertical lines relate days when many animals died with the corresponding temperature and barometric pressure. These lines are numbered to facilitate the discussion of events happening on the designated dates.

Arrow 1 connects the elevated peak of the barograph, low temperature, and points to the death of 4 of 10 injected rabbits. This is a day when cold air masses were passing and primarily a pressor phase existed in the animals. Line 2 shows death of 2 rabbits on the day of the injection of neoarsphenamine. At this time the meteorologic condition has been reversed in comparison with the previous day, the barometric pressure was low, and the temperature had reached a crest. Line 3 points to another 2 deaths on the same day of administration of drug; on this day the meteorologic conditions were quite different

from those of the previous day, namely, the temperature had fallen and the barometric pressure had increased rapidly. On line 4 four deaths occurred in three groups of animals previously injected. The lowest temperature and the highest barometric pressure for the period existed at this time.

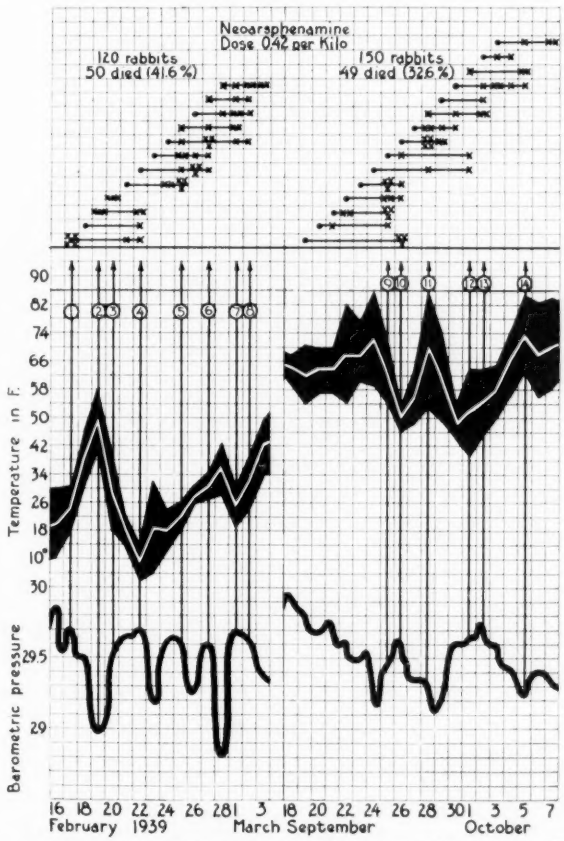

Chart 1.—Mortality protocol of rabbits injected with neoarsphenamine. Spring and autumn groups. Meteorogram below. Upturned arrows indicate days of increased mortality.

Line 5 points to 8 deaths that occurred in five groups of the previously injected animals. One death occurred in the animal injected on this day; at this time the crest of the polar air mass had just passed, and barometric pressure was falling. On line 6 there were 7 deaths in five groups, one of the deaths occurring on the day of injection. The temperature was slowly rising, but the barometric pressure reached its peak, sharply rising from the previous day. Line 7 points to 7 deaths in five groups. There was a sudden fall in temperature and a sharp rise in barometric pressure. From this day on there were no more daily injections. On March 8 there were 5 deaths in four groups.

Autumn Group.—In the autumn group the death rate was lower and, as can be seen in Chart 1, the temperature was on the whole much higher and the barometric pressure varied less. Yet during this period there were certain days on which the deaths varied in comparison with the others.

Line 9 shows 9 deaths in four daily groups; a polar front passed at this time, the temperature began to fall and the barometric pressure increased. On

the next day (line 10) 6 deaths occurred in four groups. A temperature minimum was reached and the barometric pressure crest occurred. Line 11 points to 8 deaths which occurred in four groups, one death occurring on the day of injection. Though the barometric pressure on this day declined slowly, the difference between the maximum and minimum temperatures was very great, indicating the disturbed atmospheric conditions at this time. Line 12 shows only 3 deaths occurred in three daily groups, but one death occurred on the day of injection. The temperature was increasing and the barometric pressure was still increasing. On line 13 there are shown 4 deaths in three groups, and a slowly rising mean temperature; barometric pressure reached its highest peak. Line 14 shows 4 deaths in three groups and a large difference between maximum and minimum temperatures, and the lowest barometric pressure in six days. No injections were made on this day.

The following conclusions may be drawn:

1. In late winter the neoarsphenamine is more toxic for rabbits in comparison with the same dose given under similar conditions in the autumn.

2. Toxic effects of neoarsphenamine are more marked on the days when the temperature is low and the barometric pressure is high (polar air mass).

3. Neoarsphenamine is also more toxic when the temperature is unusually high and the barometric pressure falls abruptly.

4. It is also somewhat more toxic when the difference between the maximum and minimum temperatures is great.

5. Presumably atmospheric interfaces are associated with greater susceptibility on the part of the animals. The aforesaid is true for deaths occurring immediately after the injection of neoarsphenamine as well as for delayed deaths.

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II. DAILY VARIATIONS IN THE TOXICITY OF NEOARSPHENAMINE IN WHITE RATS*

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IT HAS been shown that the toxicity of neoarsphenamine varies considerably by days in rabbits.[1] Herein are presented observations on white rats. The animals were of the same breeds, approximately of the same age. The females were virgins, and in each daily group the same number of male and female animals were used. The animals were kept in an air-conditioned room (controlled temperature) under the same care. The diet consisted of dog food cubes (given daily), lettuce—three times a week, and raw meat once a week. Water was given freely.

The experiments were performed in groups. Each day the same number of animals was injected with the same dose of neoarsphenamine, which was established on the basis of an average death rate of approximately half the number of the animals injected. The dose used was 0.225 Gm. per kilogram of weight and the injection was made at the tail. The neoarsphenamine used in all these experiments was of the same lot, dissolved under similar conditions. The solution of the neoarsphenamine was never older than six minutes from the time the ampoule was opened. The injections were administered at the same time in the morning.

The experiments on the white rats were performed in two series, the first one from May to June, and the second one from September to October, 1939. One hundred animals were used in the first series, 10 rats being injected daily for ten consecutive days, beginning May 24. The second series of experiments contained 140 animals, 10 animals per day, and began on September 19. The date of injections and the date of death, as well as the maximal and minimal daily temperature and barometric pressure for these days, are presented in Chart 1.

In the upper part of the chart are recorded the days, designated by black dots, on which the rats were given the neoarsphenamine at the tail. Each cross represents a death of one animal and is plotted on the day when this death occurred. The black lines, starting from the black dot, are extended to the right, and the crosses on these lines represent the death of a single animal on a certain day, and indicate the group to which the animal belonged.

First Group.—Line 1 (May 26) shows 4 deaths in two daily groups. At this date the barometric pressure was at its crest. Line 2 (May 29) reveals 4 deaths in three daily groups. Low temperature and high barometric pressure existed. Lines 3 and 4 (May 30 and 31) show 4 deaths in three daily groups for May 30, and 6 deaths in four groups for May 31. On these two days we had noticeable atmospheric disturbance indicated by great differences between the maximum and minimum temperatures. With line 5 (June 1) 9 deaths

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were observed in five groups. The temperature was falling and barometric pressure was rising (polar air mass). On line 6 (June 2) 11 deaths in six daily groups are noted. Difference between maximum and minimum temperatures was well pronounced, and the mean temperature decreased slowly. Line 7 (June 3) indicated 14 deaths in seven daily groups. There was a fall in temperature and a sharp rise in barometric pressure (polar air mass).

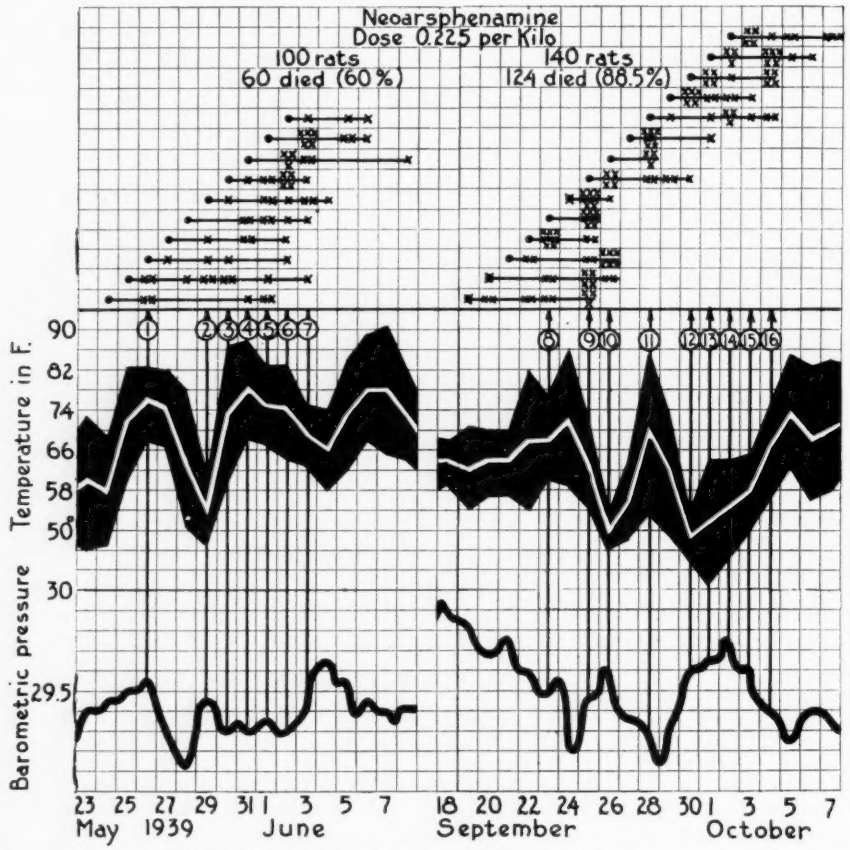

Chart 1.—Mortality protocol of rats injected with neoarsphenamine. Spring and autumn groups. Upturned arrows indicate days of increased mortality.

Second Group.—In the September to October group line 8 (September 23) indicates 12 deaths in three daily groups. On this day there was a drop in temperature and the difference between maximum and minimum temperatures was pronounced. Lines 9 and 10 (September 25 and 26) reveal the passage of a major polar air mass. Twenty-seven deaths are indicated in six groups on September 25, and 13 deaths in four groups on September 26. Line 11 (September 28) reveals 12 deaths in three daily groups. The difference between maximum and minimum temperatures on this day was large. Line 12 (September 30) falls on the day of a polar front; 6 deaths were recorded in two daily groups. Lines 13 and 14 (October 1 and 2) indicate 8 deaths in four groups, and 9 deaths also in four groups. The barometric pressure was still rising, the temperature was comparatively low, and the difference between

maximum and minimum temperatures was pronounced. Lines 15 and 16 (October 3 and 4) show 6 deaths in three groups (October 3), and 12 deaths in four daily groups (October 6). On these days the temperature was rising and barometric pressure was falling. Disturbance is indicated by the large differences in maximum and minimum temperatures.

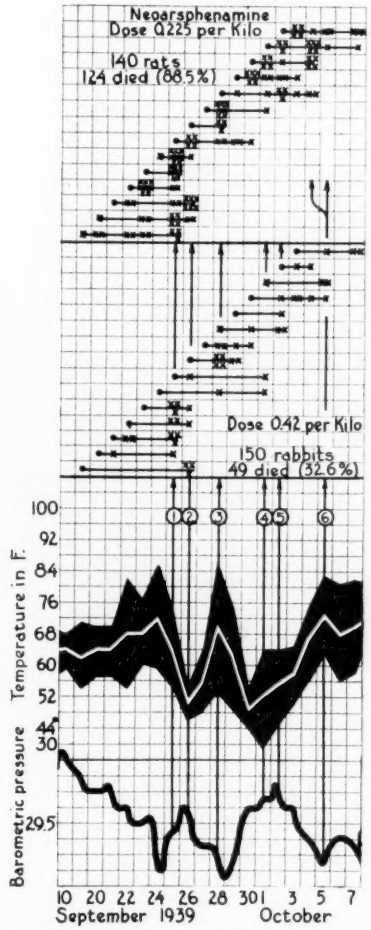

Chart 2.—Graph to illustrate similar trends in mortality for the autumn rat and rabbit groups.

From these observations the following conclusions may be drawn:

1. Neoarsphenamine is more toxic to white rats when meteorologic disturbances at the time of its administration are more pronounced.

2. The most toxic effects of neoarsphenamine in white rats are found to be on the days when the temperature is low and the barometric pressure is high (passing of polar air masses).

3. Neoarsphenamine is more toxic on the days when the temperature rises rapidly and the barometric pressure falls abruptly.

4. Neoarsphenamine is also somewhat more toxic when the difference between the maximum and minimum temperatures is great.

5. Increased toxicity holds for the deaths occurring immediately after the injection of neoarsphenamine as well as for delayed deaths.

Rabbits and Rats of September-October Group.—During the experimentation from September to October, 1939, it was possible to give the injections to rabbits (Nedzel) and white rats simultaneously. The results are presented in Chart 2. It becomes quite evident that the days when a larger number of deaths occurred among rabbits, a larger number of deaths among the rats also occurred (lines 1, 2, 3, 4, 5, and 6).

The following conclusions seem justified:

1. Similar environmental situations increase the toxicity of neoarsphenamine for different kinds of animals (in this case, rabbits and white rats).

2. Some days when rabbits survived or died in smaller numbers, were more fatal for the rats because of their greater susceptibility to the neoarsphenamine.

3. The days on which rats survived or died in a small number coincided with similar lessening of deaths for rabbits.

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A RESPIRATION TAMBOUR WITH SOME UNIQUE FEATURES*

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MANY varieties of respiration tambour are now in use in teaching and research laboratories. It is safe to say, however, that there is room for improvement in this type of apparatus. In teaching laboratories the simply constructed apparatus is preferred. Because it is handled by inexperienced hands, a breakdown of the apparatus occurs frequently in the middle of an experiment.

A respiration tambour should give satisfactory recordings from large animals, such as dogs, as well as small animals, such as rabbits. A correct recording from any kind of pneumograph or stethograph is advantageous. The cost of the apparatus should be reasonably cheap. The apparatus herein described meets these requirements. In addition it is easily adjusted. This eliminates confusion and may save the experiment.

This tambour is shown in Figs. 1, 2, and 3. The air cup *P* is cone-shaped, 42 mm. by 35 mm. This shape appears to eliminate some vibration of the writing lever. It produces a satisfactory record on the kymograph paper even with small animals, from which a good record is sometimes hard to obtain with many tambours.

An important adjustable feature is a flat piece, 35 mm. by 7 mm. by 4 mm.: *A*, Fig. 1, with one end fastened to the bottom of the tambour cup with an adjustable screw *B*. This enables arm *A* to be swung into any horizontal position. The opposite end of *A* is of different dimensions, 8 mm. by 7 mm. by 7 mm. Through a hole in this end, a vertical rod *C*, 80 mm. by 4 mm., is fitted.

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The bottom end of this rod is smaller in diameter than the top, the lower 30 mm. being 3 mm. in diameter instead of 4 mm., as it is at the top. This prevents the rod from slipping down in its entire length when being adjusted. A screw, *D*, in the end of the bottom piece, *A*, clamps the rod in the desired position. This rod adjusts the position of the writing lever in the horizontal plane. The top of the rod *C* is square; *E*, Fig. 2, measures 8 mm. by 7 mm. by 7 mm. Through a hole in this end is a horizontal rod *F* (Fig. 2), 70 mm. by 3 mm. One end of rod *F* is threaded and holds the lever assembly *G*. Movement of the lever assembly *G* about the axis of rod *C*, and by means of adjustment of the distance of this assembly from *E* accomplishes the change of position of the lever assembly to either side of the assembly *I*, which transmits the force from

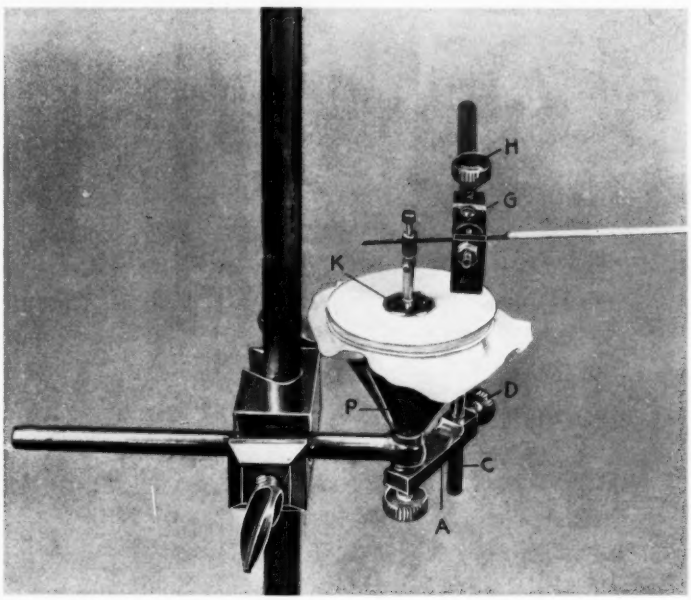

Fig. 1.—Lever joint is placed in front of the connection pin, reversing the direction of the writing level from the usual arrangement.

the tambour to the writing lever, *J*. Rod *F* is clamped in position with screw *H* on the end of vertical rod *C*. This change in position of the lever assembly *G* with reference to the fulcrum assembly *I*, enables one to change the direction of the movement of the lever. And in this way an adjustment to any type of pneumograph or stethograph can be made to obtain an upward movement of the record to represent inspiration. So far as we know this is a unique feature of this apparatus.

The footplate *K*, which is cemented to the rubber diaphragm, is connected to *I* by means of a swivel joint. This arrangement prevents the tearing loose of the footplate from the diaphragm when adjustments of the writing lever are made. This feature is also unique and can be used on any kind of tambour.

The writing lever *J* consists of an ordinary aluminum or straw heart lever. It is attached at *L* to the permanent portion of the lever assembly. It is, however, attached to the pivot *M* of the lever assembly with a small screw *N* set in a block *O*, in the same manner as an open-jawed support clamp. By this means,

the lever may be moved not only backwards and forwards but it may also be lifted out of the block *O* from any position. The block *O* which holds the lever is also attached to pivot *M* by the same arrangement.

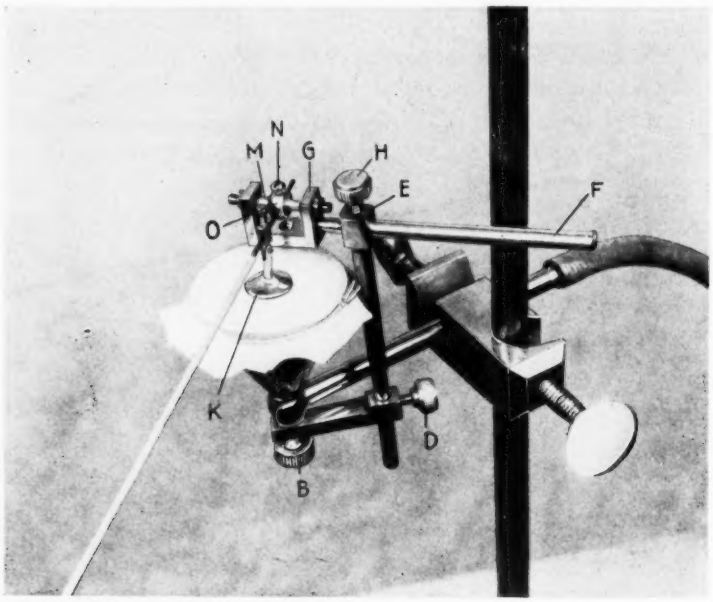

Fig. 2.—Writing lever turned in one of the many directions in which it can be placed.

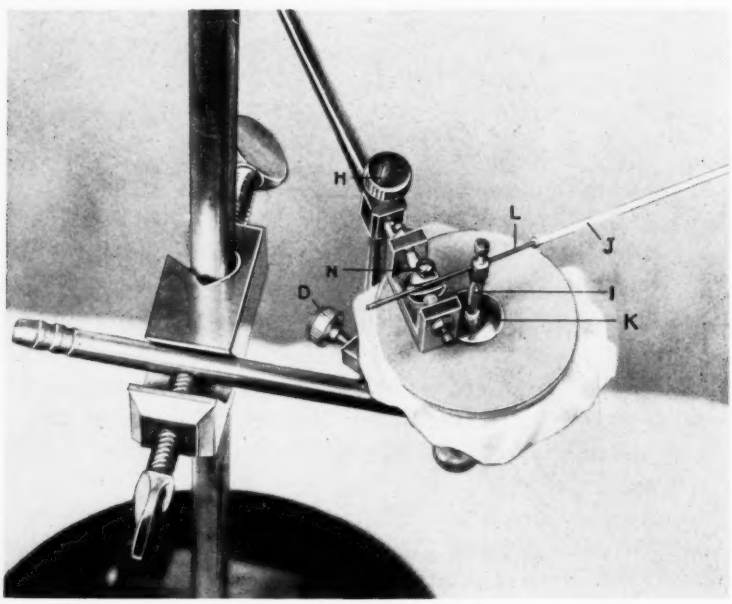

Fig. 3.—The lever joint is placed behind connection pin as in the usual tambour arrangement.

The apparatus is made of sheet copper and brass rod which are nickel-plated. The cost is reasonable and the apparatus is easily made by any competent mechanic.

THE SEDIMENTATION RATE IN GOUT*

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Cleveland, Ohio

THE sedimentation rate of erythrocytes has been investigated thoroughly in most rheumatic states, but it has not been studied to any extent in gout. Hill,[1] and Gibson and Kersley,[2] report the sedimentation rate in a fairly large series of cases of gout. A few other reports, representing small series of cases, have appeared. Here, however, the records relative to the sedimentation rate in gout have been presented only as interesting observations incidental to the study of joint disease as a whole.

PROCEDURE

We have studied the sedimentation rate in 100 consecutive cases of gout; 21 of these were patients with tophaceous gout and 79 were of the pretophaceous type. The diagnosis in the latter group was established by careful consideration of the history and physical and laboratory findings. The patients also were studied with reference to the activity of the disease at the time the examination was made. The level of uric acid in the blood was likewise determined.

For comparison, the sedimentation rate was measured in 50 apparently normal persons, in 100 consecutive cases of rheumatoid arthritis, and in 100 consecutive cases of osteoarthritis.

The erythrocyte sedimentation rate was determined by the method of Rourke and Ernstene[3] in which correction is made for the red blood cell volume. The upper limit of normal is considered 0.45 mm. per minute. The blood uric acid determination was made by the Morris-Macleod[4] technique and 2.5 mg. per cent was taken as the upper limit of normal.

RESULTS

The sedimentation rate in 100 cases of gout in all stages of activity ranged from 0.22 to 2.30 mm. per minute, with an average rate of 0.94 mm. per minute; 87 per cent of the patients showed an increase in the rate above the upper limit of normal of 0.45 mm. per minute (Table I).

The average sedimentation rate found in the normal group, in the group with osteo-arthritis, and in the rheumatoid group was 0.24, 0.51, and 0.98 mm. per minute, respectively. These are shown and compared with the gout group in Table II.

In Table III is recorded the average sedimentation rate found when the cases of gout were classified according to the activity of the disease. The average

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blood uric acid for each of these groups is noted also. The average rates found are 1.13, 0.87, 1.21, 1.02, and 0.68 mm. per minute for the acute, acute subsiding, chronic progressive, chronic, and inactive groups, respectively. The average blood uric acid values are 3.8, 3.7, 3.3, 3.9, and 4.0 mg. per cent for each group given in the same order.

TABLE I

SEDIMENTATION RATE IN GOUT

Sedimentation rate in millimeters per minute	Under 0.46	0.46-0.60	0.60-0.80	0.80-1.00	1.0 Plus
Number of cases	13	8	16	15	48
Per cent	13.0	8.0	16.0	15.0	48.0

Average sedimentation rate	0.94 mm. per minute
Highest	2.30 mm. per minute
Lowest	0.22 mm. per minute

TABLE II

COMPARISON OF SEDIMENTATION RATES IN NORMAL PERSONS, ARTHRITIS, AND GOUT

DIAGNOSIS	NUMBER OF CASES	AVERAGE SEDIMENTATION RATE
Normal persons	50	0.24
Osteo-arthritis	100	0.51
Rheumatoid arthritis	100	0.98
Gout	100	0.94

TABLE III

ACTIVITY OF DISEASE, AVERAGE SEDIMENTATION RATE, AND AVERAGE BLOOD URIC ACID IN GOUT

	ACUTE	ACUTE SUBSIDING	CHRONIC PROGRESSIVE	CHRONIC	INACTIVE
Number of cases	45	26	2	9	18
Per cent	45.0	26.0	2.0	9.0	18.0
Average sedimentation rate in millimeters per minute	1.13	0.87	1.21	1.02	0.68
Average blood uric acid in milligrams per cent	3.8	3.7	3.3	3.9	4.0

DISCUSSION

Since the original investigations of Fahraeus[5] on the sedimentation rate of erythrocytes, much interest has been shown in this property of the red blood cells. Many studies in rheumatic disorders have shown the sedimentation rate to be consistently elevated in rheumatoid arthritis, with only slight elevation in the osteo-arthritic type, probably not out of proportion to the advanced age of the persons in whom it occurs. The findings of others have been essentially in agreement with the average values cited in Table II, taken for comparison with our average values for gout.

In the occasional case of gout studied with reference to the sedimentation rate, there has usually been a tendency to an increased velocity of settling of the erythrocytes, but the striking uniformity in this increase in the rate and the magnitude of this increase probably has not been properly appreciated. Kahlmeter,[6] in a discussion on the sedimentation rate in rheumatic disorders,

reported observations with respect to this in 6 cases of gout, 3 active and 3 chronic. He found the sedimentation rates definitely elevated in the active cases with rates only slightly above normal in the chronic cases. He does not indicate clearly whether the cases in the latter group represent chronically active disease or whether they are merely cases of long duration with intermittent clinical activity. Hench[7] states that the sedimentation rate is elevated in gout but he does not further elaborate on this statement of fact. Hill,[1] reporting his observations on 93 cases, observed that the sedimentation rate varies considerably in gout, being markedly increased only during acute attacks and returning quickly to normal following the acute episode. Gibson and Kersley[2] found the erythrocyte sedimentation rate to be increased in 59 per cent of 68 cases of gout they studied. It is their belief that the sedimentation rate measures the factor of severity of the local tissue reaction.

Although the average rate found here for the 100 consecutive cases was 0.94 mm. per minute and an elevation above normal occurred in 87 per cent of the patients, it should be emphasized that the sedimentation rate may be found to be within normal limits in certain instances. Thirteen of the 100 patients had rates of 0.45 mm. per minute or less. However, in each instance the gout was either subsiding after an acute exacerbation or was classified as inactive. On the other hand, many cases of inactive and subsiding acute gout were seen in which the readings were decidedly elevated.

The sedimentation rate tends to reflect in part, at least, the activity of the disease. This interesting observation is drawn from examination of Table III, where it is seen that the highest average values in the common type of intermittently active gout occurs in the group representing the acute phase of the disease. As the process becomes less active, the average rate is reduced, as noted in the average values found in the acute subsiding and inactive groups. When the disease tends to be of a progressive nature, the sedimentation rate reaches its highest level. The observations made in this particular group, however, represent only 2 cases and are, therefore, subject to criticism. The average value found in the chronic group is about as would be expected, being neither extremely high nor as low as in the inactive group. The shift in the sedimentation rate noted with respect to the activity of the disease is, of course, not peculiar to gout. Essentially the same reaction is encountered in rheumatoid arthritis.

There is no adequate explanation for the elevation of the sedimentation rate in gout at the present time. Infection is certainly not a factor, for careful survey of these individuals for chronic foci has usually proved unsuccessful. Treatment or removal of infected teeth, tonsils, sinuses, or prostate glands had been carried out in 45 per cent of these cases before they came under our observation, without affecting the course of the disease. Gilligan and Ernstene[8] have demonstrated that the plasma fibrinogen level is the major factor in controlling the sedimentation rate. Assuming an increase in the plasma fibrinogen in gout, one may offer a possible explanation on a basis of faulty intermediary protein metabolism or perhaps on the basis of impaired liver function. A functional disturbance in the liver in gout does not seem unlikely, since these patients

are almost uniformly obese and are notorious for their indiscretion regarding diet and the use of alcohol.

An examination of the data, as shown in Table III, fails to reveal any parallelism between the sedimentation rate and the level of the blood uric acid which reflects in part, at least, the extent of metabolic dysfunction.

SUMMARY

1. The sedimentation rate was found to be elevated above 0.45 mm. per minute in 87 per cent of 100 cases of gout. The average rate for the series was 0.94 mm. per minute. The upper limit of normal by our method is 0.45 mm. per minute.

2. For comparison the sedimentation rate in 50 apparently normal persons, 100 cases of osteo-arthritis, and 100 cases of rheumatoid arthritis was determined. The average rates found were 0.24, 0.58, and 0.98 mm. per minute, respectively.

3. The sedimentation rate varies with the activity of the disease, being lower in acute subsiding cases than in acute cases, and reaching its lowest level in inactive cases. It reaches its highest level in the chronic progressive type of gout and an intermediate level in the simple chronic type of the disease.

4. There is no apparent correlation between the sedimentation rate and the degree of metabolic dysfunction, as indicated by the level of the blood uric acid.

5. The sedimentation rate may at times be of value in the differential diagnosis of gout, osteo-arthritis, traumatic arthritis, bursitis, tendonitis, fibrositis, and periarthritis.

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THE ROLE OF AUTO-ANTIBODIES IN THE SERODIAGNOSIS OF SYPHILIS*

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THE history of the serodiagnosis of syphilis and the speculation as to the nature of the constituent of serum that reacts with tissue lipids in the present-day serodiagnostic tests for syphilis have been summarized by Eagle.[9, 10] From the 1937 summary[9] it seems controversial as to whether reagin is an antibody to lipid haptens of the host, liberated at foci of infection and activated by spirochetal protein, or an antibody to the spirochete which contains a lipid related to those found in mammalian tissues (and in vegetables,[36] as well as possibly in other bacteria and protozoa, the so-called ubiquitous lipid). Recent work with the spirochetal complement-fixation test[10] seems to indicate that the spirochete of syphilis stimulates the production of at least two *separate* antibodies, (a) antilipid and (b) antispirochetal, both in response to substances contained in the spirochete.

With no thought of disparaging this admirable work, but rather with the hope of emphasizing its potential significance, exception must be noted to the implication of the repeated statement that "there seems no a priori reason why spirochetal protein should uniquely activate the lipoidal tissue hapten and thus initiate antibody production, while in other conditions in which there is just as much tissue destruction and in which some other bacterial protein is available as the activator, the Wassermann remains consistently negative."[9, 10] While there is indeed no apparent "a priori reason," the implication that serodiagnostic tests for syphilis remain consistently negative in other conditions is not beyond question.† From the reports of biologically positive reactions in certain nonsyphilitic diseases in man[1, 6, 7, 9, 25, 26, 28, 32-34] and lower animals,[24] the occurrence of positive reactions in apparently normal lower animals[9, 13, 31] and the possible occurrence of reagin in normal human serum[2] all constitute evidence that must not yet be disregarded.

Rosebury[12] pointed out that, theoretically, spirochetal protein is not necessary for the activation of autogenous lipoidal haptens, and Kahn[18] emphasized the possibility that in the presence of tissue destruction lipid protein combinations may be liberated similar to those liberated in syphilis. He further pointed out that in rare instances traces of tissue proteins and lipids liberated in the process of metabolism might be sufficient to stimulate antibody production against lipids.

The production of auto-antigens within the living body of an experimental animal (guinea pig) by application of the physical agents of heat or cold has recently been reported by Karady[19] but has not been confirmed.

Reference has already been made to the occurrence of positive reactions in man in certain diseases other than syphilis. Particular attention is called to the report of Taoka[33] that positive serologic reactions were found in 24 per cent of 100 patients with carcinoma of the cervix uteri as compared with only 10.38 per cent of 106 general in-patients; also Stojalowski[32] found that of 830 cases studied by post-mortem examination there were 15 with no post-mortem sign of syphilis, which had been diagnosed and treated for syphilis. In 5 cases serologic tests were not done; in 3 cases serologic reactions were negative; and, in 7 cases serologic tests were done only in the moribund stage. In correlating the post-

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†Since this was written the following papers have been published:

Kahn, R. L.: A Serologic Verification Test in the Diagnosis of Latent Syphilis, Arch. Dermat. & Syph. **41**: 817, 1940.

Moore, J. E., Eagle, H., and Mohr, C. T.: Biologic False Positive Serologic Tests for Syphilis, J. A. M. A. **115**: 1602, 1940.

mortem histologic diagnoses with the serologic findings he found that: of 2 cases of carcinoma 2 were positive; of 5 cases of tuberculosis 2 were positive; and of 5 cases of myocarditis 2 were positive.

It has been reported that positive serologic reactions for syphilis have been induced in rabbits by the injection of various preparations. These include: killed cultures of *T. pallidum*;[20, 21] aqueous extract of syphilitic liver;[5, 11, 16] alcoholic extract of syphilitic liver[11] and syphilitic rabbit's testicle;[14] mixtures of lipid extracts from various normal human, rabbit, or beef organs with some foreign protein;[4, 8, 15, 16, 30, 35] a mixture of lecithin and hog serum;[27] washed floccules from positive reactions of human serum from syphilitic patients[3, 8, 29] and from malarial patients,[29] and of pooled bovine serum,[29] and syphilitic rabbit serum;[3] aolan;[27] and emulsions of hamster tissues.[28] Positive reactions in rabbits have also been noted as a result of experimental infection with bovine tubercle bacillus,[24] and have been induced in human beings by the injection of killed cultures of *T. pallidum*,[22] and killed trypanosomes.[23, 38]

Reports of unsuccessful attempts to produce artificial positive reactions in rabbits include the injection of alcoholic extracts of normal human heart and normal infant liver;[11] alcoholic extracts of (syphilitic ?) liver;[16] alcoholic extracts of normal rabbit's testicle;[14] turpentine, dilute alcohol, and *b*-tetrahydronaphthylamine;[24] and floccules from positive tests on normal rabbit serum,[8] and human leprous serum (one trial only).[29]

Because of the incidence of positive reactions in rabbits,[9, 13, 24, 31] it is difficult to interpret all of these reports. However, they seem to indicate that it is possible artificially to induce positive serologic reactions in rabbits by various methods, many of which in no way involve either *T. pallidum* or any specific derivative of it.

With these things in mind it appeared that the auto-antibody theory might account for positive reactions with lipid antigen in the absence of syphilis, and that it might be susceptible to investigation by animal experimentation.

EXPERIMENTAL

Materials and Methods.—Rabbits, after preliminary serologic study, were variously treated in attempts (a) to produce pathologic changes characterized by chronic inflammation and degenerative changes simulating the essential lesions of syphilis while observations of the reagin titer were made at frequent intervals; or (b) to stimulate or to augment the production of reagin by the injection of various extracts of homologous tissue or preparations of autogenous serum.

Necropsy has been performed upon these animals to observe any correlation between the serologic findings and the pathologic changes.

The serologic examination included the Kahn Standard, Kline Diagnostic, and the Mazzini flocculation tests; quantitative tests were done whenever a four-plus reaction was encountered. Complement-fixation tests were attempted, but the high incidence of anticomplementary reactions rendered the results almost worthless. For a time the sera were heated for thirty minutes at 62° C. instead of 56° C., in an attempt to reduce the incidence of these anticomplementary reactions. It was observed that heating at this higher temperature reduced not only the anticomplementary activity but also the reagin titer; consequently, this deviation from the regular technique was soon abandoned.

Incidence of Serologic Reactions in Untreated Rabbits.—The sera from 18 rabbits were tested after heating at 56° C. for thirty minutes; 2 were Kahn negative, 2 were Kline negative, and 6 were Mazzini negative. The reactions of the others ranged from a one-plus reaction to a titer of 5. Three rabbits were not tested with the complement-fixation test and 2 of them were anticomplementary in all reacting dilutions; of the 13 on which readings were obtained, 7 were negative.

The sera of 10 rabbits were tested after heating at 62° C. for thirty minutes; 10 were complement-fixation-negative, 8 were Kahn negative, 8 were Kline negative, and 8 were Mazzini negative. The same 2 rabbits reacted in the Kline and Mazzini tests; the 2 rabbits which reacted in the Kahn test were negative to all other tests.

From these groups animals were chosen for further study.

Variation of Serologic Titer in Untreated Rabbits.—Of the 8 rabbits first chosen for study all but one showed considerable variation of reagin titer, as demonstrated by the flocculation tests; one animal gave consistently negative results with all tests. A study of the records of these animals reveals that (a) upon repeated examination the various techniques did not show similar variation of titer, one rabbit excepted; (b) upon repeated examination with a given technique the various animals did not show a uniform variation from the titer of the previous examination with the same technique; and (c) in general, the Mazzini technique in repeated examinations of a given animal seemed to show a lower titer and less variation in titer than did either the Kline or Kahn test; one rabbit represents an exception to this observation and exhibited a lesion (echinococcal cyst) not found in the other animals.

While the possibility of some daily variation in the sensitivity of the various antigen emulsions cannot be completely ruled out, these observations are considered as sufficient evidence for the belief that this factor does not account for the variations in titer observed under treatment.

PATHOGENIC AND IMMUNOLOGIC PROCEDURES

1. *The injection of flocculate from positive human serum.* In order to secure a preliminary observation as a basis of comparison, a female rabbit, which had shown clearly negative results over a period of six weeks, was given weekly intravenous injections of washed flocculate from mixtures of Mazzini-antigen suspension and positive human serum. A total of 9 injections of floccules from 0.6 ml. of four-plus serum was given.

One week following the third injection the Kahn test showed a one-plus titer; the following week this had increased to a two-plus. Just before the fifth injection the Kline, Mazzini, and complement-fixation tests showed partial positives for the first time. The Mazzini test gradually rose to a titer of 10 before the eighth injection. During the following week it dropped sharply to one-plus.

2. *Intra-abdominal transplant of rabbit's aorta.* A freshly removed rabbit aorta was placed in the peritoneal cavity of a female rabbit. No abnormalities were observed, and clinical recovery was uneventful. The Kahn titer rose from

one-plus to two-plus two weeks after the operation, and was maintained at this level until the seventh week when it dropped again to one-plus. The Kline titer was slightly diminished during the first four weeks, returned to one-plus in the fifth week, rose slightly in the sixth week, and returned to one-plus in the seventh week. The Mazzini titer was slightly elevated in the fourth, fifth, and sixth weeks, and again dropped to negative in the seventh week. Necropsy in the eighth week showed an encysted granulomatous mass enclosing the transplanted aorta.

3. *Ligation of the splenic pedicle.* A female rabbit was subjected to laparotomy, at which time the splenic pedicle was ligated. No abnormalities were observed, and clinical recovery was uneventful.

The Kahn titer rose to three-plus in ten days and to four-plus in thirty-one days after the ligation. During the next week it dropped to one-plus. The Kline and Mazzini tests showed no significant variation of titer. The complement-fixation test was one-plus two and one-half weeks after surgery, but later examinations were again negative. Necropsy in the seventh week revealed replacement of the greater portion of the spleen by a nonspecific granulomatous mass exhibiting central necrosis resembling caseation.

4. *Injection of homologous tissue preparations.* The heart, aorta, liver, brain, and psoas major muscles of an untreated female rabbit were dehydrated in repeated changes of acetone. The dehydrated tissue was finely chopped and mixed for the preparation of the various extracts.

A. *Aqueous extract of homologous tissues*

Three grams of this dehydrated rabbit tissue were mixed with 50 ml. of 0.85 per cent sodium chloride solution and shaken mechanically for an hour. This mixture was filtered, the residue was discarded, and merthiolate, 1:40,000, was added. Two rabbits were given weekly intra-abdominal injections of this aqueous extract, starting with a dose of 1 ml. and increasing to 3 ml. in the seventh week. One rabbit showed no significant variation in serologic titer during eight weeks, nor were significant changes found at necropsy. The other rabbit showed some fluctuation in the Mazzini titer, and a gradual increase in the Kahn titer from negative at the end of the second week to three-plus at the end of the eighth week. This latter rabbit at necropsy exhibited a splenic lesion which consisted of a cystlike yellow mass containing dirty dark-brown cheesy material. Microscopically, it could be seen that the yellow color was due to yellow droplets in phagocytic cells which composed the inner portion of the "cyst" wall. These cells appeared to be large mononuclear cells, which in some areas exhibited a loss of cell outline and resembled syncytium. Multinuclear cells of the foreign-body type were seen. The etiology of this lesion remains obscure.

B. *Lipid extract of homologous tissues*

Fifteen grams of the dehydrated rabbit tissues were treated to obtain ether-soluble acetone-insoluble and ether-insoluble alcohol-soluble fractions which were mixed in alcoholic solution and portions used for injection after being quickly added to an excess of 0.85 per cent sodium chloride solution.

Two rabbits got weekly intra-abdominal injections of this extract, 0.5 ml. in each of the first four doses and 1.0 ml. in each of the last three. One of these rabbits after negative preliminary tests exhibited a gradual rise to a two-plus Kahn and a one-plus Kline and Mazzini; no significant lesions were found at necropsy. The other rabbit exhibited preliminary reactions varying between three-plus and a titer of 10. Under treatment the Mazzini titer was constant at four-plus; the Kline fluctuated from a level of three-plus; and the Kahn was at first diminished to a two-plus and then rose to a four-plus level. At necropsy an echinococcal cyst was found.

C. *Protein derivatives of rabbit tissues (peptic metaprotein)*

The residue of the rabbit tissues after ether and alcohol extraction as already described was treated with 150 ml. of 0.3 per cent hydrochloric acid and approximately 0.25 Gm. of pepsin. After ten minutes at 37° C. a small portion was separated, while the remainder was permitted to stand for four hours at this temperature. The material was filtered, neutralized with sodium hydroxide, and heated to boiling. The products of the ten-minute and the four-hour incubations were mixed. This preparation yielded positive biuret and xanthoprotein reactions; Millon's reaction was negative. A precipitate formed upon the addition of ammonium sulfate, but boiling did not cause precipitation. Merthiolate, 1:40,000, was added.

Two rabbits were treated with weekly intra-abdominal injections of this preparation, the doses increasing from 1.0 to 3.0 ml. in the ninth week. Both animals showed slight increases of the Kahn titer without significant change in the results of the other tests. Neither animal showed significant lesions at necropsy.

D. *Recombined lipoid and peptic metaprotein from rabbit tissues*

A portion of the peptic metaprotein extract was treated by adding to it, drop by drop, during vigorous agitation, the lipid extract (B) until a preparation resembling clear yellow blood serum was obtained.

Six rabbits were treated with weekly injections of this preparation in doses increasing from 1.0 to 3.0 ml. in the ninth week. Two showed very slight transient increases of titer. Necropsy revealed in one cysticercoid cysts of the omentum and in the other a 5 mm. encysted granulomatous abscess located retroperitoneally. One showed fluctuation of the previously constant Kahn titer in an elevated range; necropsy revealed no significant lesions. Two showed primary diminution and secondary elevation of titer, most marked in the Kahn, but also evident in the other flocculation tests; necropsy revealed no significant lesions in one of these, while in the other occasional small areas of patchy necrosis were present in the renal cortex. One, after a previously negative reaction, showed a definite rise of titer, evident in all the tests and comparable to that seen in the rabbit injected with flocculate from human syphilitic serum. Aside from blindness, probably of glaucomatous origin, this rabbit exhibited no significant lesions.

5. *Injection of heated autogenous serum.* Two rabbits were treated with autogenous serum which had been heated one hour at 56° C. The dosage was

0.5, 0.75, 1.0, 1.0, 1.0, and 1.0 ml. at weekly intervals. In one rabbit these doses were reinforced with 0.2 ml. of a 5 per cent suspension of collodion particles prepared by the method of Zozaya.[37] Both animals showed a gradually increasing Kahn titer during the first four weeks, after which the titer dropped in each case. The Kline and Mazzini titer gradually decreased during the period of treatment. Neither rabbit exhibited significant pathologic changes at necropsy.

DISCUSSION

The production of a positive serologic reaction following the injection of flocculate from human syphilitic serum has been frequently reported and was readily confirmed. The fall of titer that occurred during the eighth week while the injections were still in progress does not seem to have been previously reported. Neither has the apparent partial specificity of the antibody produced for the particular antigen flocculate been emphasized.

The pathogenic procedures of aortic transplantation and splenic ligation were each successful in producing local lesions of the desired type. A somewhat similar lesion occurred, apparently spontaneously, in one of the rabbits treated with aqueous extract of rabbit tissues and may account for the changes in titer observed in this animal.

It is probably apparent that among the various extracts of rabbit tissues the lipid-metaprotein preparation constituted the real test antigen, while the other preparations were employed as controls. In connection with the apparent slight antigenic action of the lipid extract it should be mentioned that its freedom from proteins is probably questionable. The colloidal state in which the lipid was injected might possibly tend to increase its antigenicity.

While with the exception of one rabbit treated with lipid-metaprotein preparation these changes are not of convincing magnitude, they are all compatible with the auto-antibody theory and are best explained by it. Furthermore, the changes in human subjects where the auto-antibody mechanism may be the responsible factor are usually of similarly slight degree.

The significance of the spirochetal complement-fixation test, especially when done after preliminary absorption with lipids, becomes even greater in the face of such an auto-antibody theory.

SUMMARY

Attempting to demonstrate evidence which would support or controvert the auto-antibody theory in accounting for nonspecific reactions with lipid antigens in serodiagnostic tests for syphilis, rabbits were subjected to various treatment intended to stimulate or to augment the production of reagin by nonspecific and noninfectious means.

1. Granulomatous lesions were produced in 2 rabbits by surgical procedures; both animals exhibited slight elevations of Kahn titer between the second and seventh weeks.

2. Of 6 rabbits treated with a lipid-metaprotein preparation, 2 showed very slight transient increases of titer, one showed fluctuation of the previously constant Kahn titer in an elevated range, 2 showed primary diminution and

secondary elevation of titer, most marked in the Kahn but also evident in the other flocculation tests; one, after a previously negative reaction, showed a definite rise of titer, evident in all tests and comparable to that seen in the rabbit injected with flocculate from human syphilitic serum.

3. Of 2 rabbits treated with heated autogenous serum, both exhibited elevation of the Kahn titer during the first five weeks.

4. Two rabbits were treated with aqueous extract of rabbit tissues. One showed no definite serologic change. The other showed a rising Kahn titer but was found to have a splenic lesion, possibly a granuloma due to the injected lipids.

5. Two rabbits were treated with a lipid extract of rabbit tissues; one showed a very slight transient increase of titer, most evident in the Kahn test, and the other exhibited an incidental lesion, Echinococcus cyst, which is believed to have affected the serologic results.

6. Two rabbits were treated with a peptic metaprotein from "lipid free" rabbit tissues, and each showed a transient increase of titer most evident in the Kahn test but of doubtful proportions.

CONCLUSIONS

While the evidence brought forward may not be entirely convincing, it is compatible with, and is best explained by, the conception that reagin is a closely related group of antibodies and not a single chemically constant compound, and that, while the primary serologic change in syphilis may be the formation of antibodies to *T. pallidum*, the potential or actual occurrence of positive or doubtful reactions with lipid antigens in the absence of syphilis results from the formation of antibodies to the "ubiquitous lipid," either from some other infectious agent or from the tissues of the host, dependent upon the liberation of lipid haptens and protein derivatives capable of activating them.

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VITAL CAPACITY STUDIES IN THE AGED

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SINCE 1846 the estimation of the vital capacity has been used as a measure of respiratory efficiency. Various standards and tables have been set based on occupation, weight, surface area, standing height, sitting height, and chest circumference. These standards were needed for comparison, since differences were found in the ability of men with the same vital capacity to resist dyspnea. In 1846 Hutchinson[1] in his work with the spirometer used the standing height as the standard of comparison, the vital capacity varying directly with the standing height. Peabody and Wentworth[2] also used standing height measurements; they divided people into three groups, according to height, and made average vital capacity readings for each group. Dreyer[3] and West[4] used surface area standards in their work. Dreyer[3] divided people into three groups, based on physical fitness. His vital capacity figures varied from high in the active athlete and laborer group to low in those with sedentary occupations. Training, sex, and occupation all played a part in the vital capacity.

Most of the work with vital capacity has been done on adults. Observations made on the aged have been only incidental and were based on relatively few cases. Hutchinson[1] found an increase in vital capacity with age up to 30 years, and a definite decrease after 50 years. Loomis[5] stated that with advancing years the vital capacity decreased with tolerable regularity, about 1.5 cubic inches each year. He reported that a healthy adult exhaled about 1,340 cubic inches of carbonic acid per hour; in men between 60 and 80 years the amount fell to 930 cubic inches, and in very old men it diminished to 670 cubic inches. Charcot[5] found that the functions of the respiratory apparatus were weakened in old age, the amount of the carbonic acid exhaled decreased, the number of inspirations increased, and the vital capacity of the lungs was reduced. Myers[6] studied the vital capacity of 88 women and 118 men, 66 years of age and over. The average vital capacity in men decreased with age from 74 per cent to 53 per cent; in women it decreased from 52 per cent to 44 per cent. Pratt[7] studied the influence of age on the vital capacity of 100 normal men in the second to the eighth decade of life. He reported a maximum capacity in the third decade, after which it slowly decreased. Bowen[8] studied the vital capacity of 184 people between 15 and 85 years, of whom 64 were over 50 years. He found the greatest drop between 50 and 60 years. Lemon and Moersch[9] stated that age had little effect before 50 or 60 years, and only a variable effect after that age. The decrease in vital capacity, if present, accompanied the decrease in physical fitness occurring at this period of life. The decrease was due not so much to age as to the degenerative changes and to the changes in height and weight which occurred in this age group. Levy[10] reported his studies

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on the vital capacity of 110 men and 71 women, 60 to 94 years of age. In men the average vital capacity decreased from 2,980 c.c. at 60 to 65 years to 2,350 c.c. at 86 to 94 years. In women the average vital capacity decreased from 1,985 c.c. at 60 to 65 years to 1,460 c.c. at 86 to 92 years.

TABLE I

AGE	NUMBER OF MEN	AVERAGE VITAL CAPACITY	
		C.C.	PERCENTAGE OF NORMAL
40-44	4	3,150	79
45-49	7	3,200	80
50-54	50	3,000	75
55-59	73	3,000	75
60-64	126	2,900	72.4
65-69	168	2,750	70.6
70-74	171	2,700	70.2
75-79	100	2,650	69.2
80-84	36	2,600	68.8
85-89	6	2,500	68.1
90-94	3	2,400	62

The vital capacity studies in this report were made on 744 men, 40 years of age and over. These men were inmates of the New York City Farm Colony, a home for the aged. The test was done in the afternoon hours, between meals, and in the different dormitories, which are situated on the ground floor and on the second floor. The men rested on the side of their beds for thirty minutes to rule out any possible dyspnea which might be due to walking or to climbing the one flight of stairs. Each man was given three trials at the spirometer, and the highest figure was taken as his normal. Weight and sitting height determinations were made on each man. The vital capacity readings were compared with the weight and sitting height, and the tables of Myers[6] were used to determine the percentage of normal.

Table I gives the average vital capacity and percentage of normal by five-year periods. There is a decrease in the average vital capacity from 3,150 c.c. at 40 to 44 years to 2,400 c.c. at 90 to 94 years. The decrease in percentage is from 79 to 62. The average vital capacity of the group as a whole was 2,780 c.c., 71 per cent of normal. Our determinations agree closely with those of Levy.[10]

DISCUSSION

Vital capacity is the sum total of tidal, supplemental, and complemental air; it is the maximum expiration of air as measured by a spirometer after the deepest inspiration. The vital capacity decreases with advancing age. The decrease is due to physiologic conditions that occur in the aging body. Degenerative changes are the most important. The lung tissue becomes less elastic, the thoracic cage is more fixed, and the muscular power diminishes.

Pulmonary pathology decreases the vital capacity. In 5 patients with pulmonary fibrosis and silicosis, the average vital capacity was 2,040 c.c., 50 per cent of normal. The figures for this group would have been lower if we had included several men whose attempts to do the test were not accurate. The first attempt at the spirometer brought on an attack of coughing and dyspnea, and further trials had to be abandoned. In 27 men with bronchiectasis the average

vital capacity was 2,130 c.c., 55 per cent of normal. Pulmonary tuberculosis (30 men) and pulmonary emphysema (31 men) gave a similar picture, an average vital capacity of 2,400 c.c., 62 per cent of normal. The patients with pulmonary tuberculosis had the productive type of lesion, with involvement of one or both apices or upper lobes.

Blood pressure variations had no effect on vital capacity with the exception of the group, with a systolic pressure of 200 mm. and over. This group had an average vital capacity of 2,400 c.c., 62 per cent of normal. Seventy men with heart murmurs, systolic murmurs at either the mitral or aortic areas, and 27 men with auricular fibrillation had normal vital capacity readings. Physical examination revealed no evidence of decompensation in these cases.

Vital capacity studies to be of use must be interpreted correctly. A single reading is not as accurate as a series of readings; a fall in vital capacity is important. A normal vital capacity does not denote the absence of pulmonary pathology; a low vital capacity is of significance. A vital capacity of 90 per cent and over is considered normal.[11] With a vital capacity of 80 to 90 per cent, work can be done, but any increased strain will produce dyspnea. With a reduction in vital capacity to 40 to 70 per cent, only light work can be done, and a slight increase in effort will produce dyspnea. With a vital capacity of 40 per cent or less, patients are usually dyspneic even without effort. Of the 744 patients 40 years of age and over, 11 per cent had a vital capacity of 90 per cent and over; 45 per cent had readings between 70 and 90 per cent; 43 per cent had readings between 40 and 70 per cent; and 2 per cent had readings under 40 per cent. It can be concluded that the older age group can do light or moderate work, but an increase in effort would produce dyspnea.

CONCLUSIONS

1. Vital capacity determinations were made on 744 men, 40 to 94 years of age. There was a decrease in vital capacity from 3,150 c.c. (79 per cent of normal) at 40 to 44 years to 2,400 c.c. (62 per cent) at 90 to 94 years. The vital capacity for the group as a whole was 2,780 c.c. (71 per cent). The causes of the decreased vital capacity are loss of elasticity of the pulmonary tissue, the lessened mobility of the thoracic cage, and diminished muscular power.

2. Pulmonary fibrosis, pulmonary tuberculosis, pulmonary emphysema, and bronchiectasis gave vital capacity readings below normal for their age groups.

3. Compensated heart cases gave normal vital capacity readings. Lowered figures were found in men with blood pressure of 200 mm. and over.

4. A normal vital capacity does not denote the absence of pulmonary pathology; a low vital capacity is significant.

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PULMONARY ASPERGILLOSIS*

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ALTHOUGH mycotic infections of the lungs have been recognized for decades, they are still very interesting and unusually hard to diagnose because of their relative rarity. Hughes Bennett in 1842 reported the first case on record. Cases of pulmonary aspergillosis reported in the nineteenth century were made by Virchow, Dieulafoy, Chantemesse, and Widal.[1]

The species of the genus Aspergillus are predominantly saprophytic, but some are quite pathogenic. Because of its omnipresence, universal infection would undoubtedly occur were not the vast majority of this genus nonpathogenic.

Aspergillosis is produced by a moldlike fungus with a basal stem and stalk supporting a spore-bearing head. This organism seems to have a definite affinity for the pulmonary system,[2, 3] although other organs may be attacked, especially the kidneys of experimental animals.

An interesting side light on this disease is given by C. W. Dodge:

> The natives of Watusi make use of the pathogenicity of some species of *Aspergillus* in an interesting way, according to Mattlet (1924). When they wish to wreak vengeance on some one, they exhume a corpse of a person recently dead of a pneumomycosis, remove the lungs, desiccate them, and mix the powder in a banana beer. Evidently the Aspergillus is not killed by this process, and the recipient wastes away with the Aspergillosis, without suspecting the cause.[2]

CASE REPORT

H. B. G., a white male, aged 40 years, a bookkeeper by occupation, who first came under observation on April 22, 1940, had a productive cough and fever. In August, 1939, he began to run an elevated temperature, and developed a yellow tinge of the skin. He had had a cough for several years, and two months before the present examination he had noticed pain in the chest. X-ray examination (Fig. 1) of the chest on August 6, 1939, revealed

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numerous scattered areas of infiltration throughout the middle and upper lobes of the right lung, especially marked in the first and second interspaces, while the mediastinum was displaced to the right. The left lung showed scattered infiltration throughout the entire upper lobe and the upper half of the lower lobe. Diffuse fibrosis was present throughout the left lung, especially marked in the upper lobe. Both hilar shadows were increased in density, while numerous circumscribed areas of rarefaction were present in both lung fields. Pulmonary tuberculosis was diagnosed from this x-ray picture, although the sputum was negative for tubercle bacilli. The patient entered the Texas State Tuberculosis Sanatorium on October 18, 1939. Many sputum examinations before entrance to the Sanatorium were negative for tubercle bacilli, as were numerous ones made during his six months' stay at this institution.

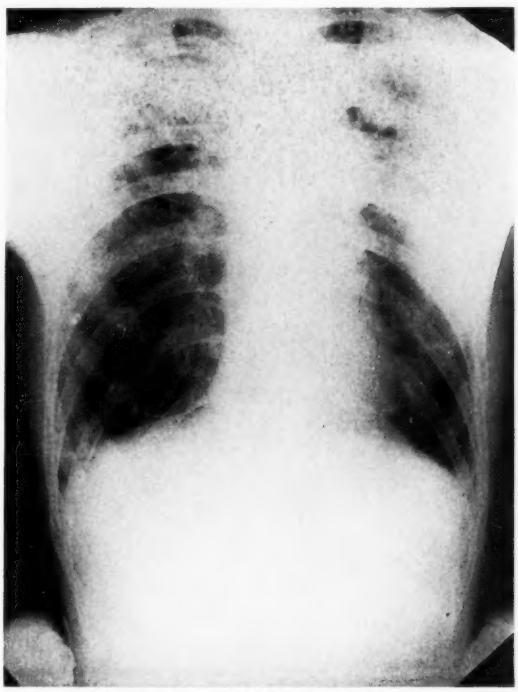

Fig. 1.—X-ray examination on Aug. 6, 1939, revealed numerous scattered areas of infiltration throughout the middle and upper lobes of the right lung. Areas of infiltration were present throughout the left upper lobe and the upper part of the lower lobe. Numerous areas of rarefaction were present in both lung fields.

Lipiodol injection of the bronchial tree revealed evidence of the presence of bronchiectasis, while a tentative diagnosis of cystic disease was made from the scattered areas of rarefaction observed in the x-ray examination.

The patient's family history was irrelevant. When a small child, he had measles, mumps, pertussis, and chickenpox. In 1918 he had influenza, and on one or two occasions since that date he has had repeated attacks. He had malaria in 1923 and a transient attack of asthma in August, 1938.

In an effort to determine the possible etiology of the asthmatic attack, the patient was referred to the allergist, Dr. B. H. Reinarz. He reported the patient to be sensitive to the following allergens: *Inhalants*, wool, mohair, and sterile mycelia. *Foods*, beef, corn, lamb, mutton, pork, veal, chocolate, goat's milk, and cow's milk.

Physical examination of all systems was negative, except as will be noted. There was blanching and thickening of the mucous membrane throughout the nose, with haziness of the sinuses, that of the left maxillary being especially marked.

Inspection of the chest showed the thorax to be poorly nourished, with all the bony landmarks prominent. There was a yellowish tinge to the skin. Decreased expansion was observed over the upper half of each hemithorax, there being a more marked restriction of expansion on the left. Increased tactile fremitus was present over the upper lobe of the left lung. Impaired resonance was elicited upon percussion of the right chest from the apex to the second rib anteriorly, and from the apex to the spine of the scapula posteriorly. Impaired resonance was present over the anterior portion of the left chest from the apex to the fifth rib, and from the apex to the angle of the scapula posteriorly. From auscultation the presence of harsh breath sounds was noted over the apex of the right lung anteriorly and posteriorly. The whispered voice was increased in intensity over the same area, but no râles were present. Increased intensity of the breath sounds was noted from the left clavicle to the fourth rib anteriorly, and over the suprascapular region posteriorly. Fine and medium moist râles were observed in these same areas.

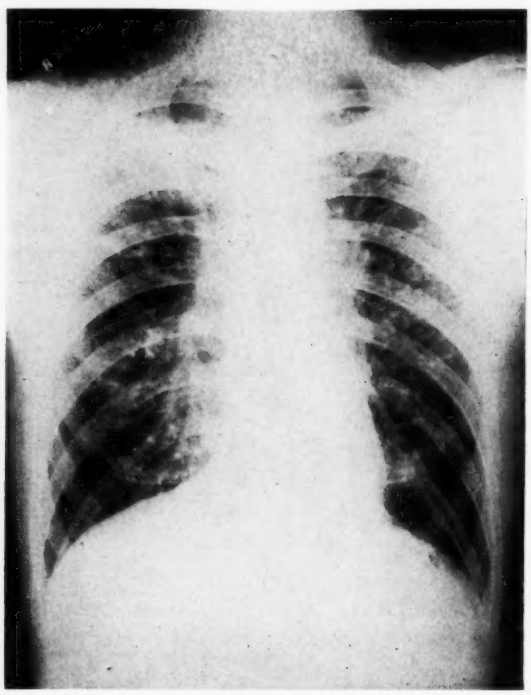

Fig. 2.—X-ray examination on April 22, 1940, showed some clearing of the infiltration in both lungs since the last examination. Fibrosis was marked in both lung fields.

X-ray examination on April 22, 1940 (Fig. 2) revealed a decrease in the degree of the infiltration observed in the right lung on previous examination. Fibrosis was still marked especially in the middle and upper lobes. The mediastinum was displaced to the right. There had been slight clearing of the infiltration in the left lung. Scattered areas of rarefaction were still present in both lungs.

The urine was negative, except for an occasional pus cell; the erythrocyte count was 3,930,000; the leucocyte count was 7,000; the hemoglobin 85 per cent (Dare); polymorphonuclear leucocytes 65 per cent; lymphocytes 20 per cent; monocytes 10 per cent; eosinophiles 5 per cent; Kahn test negative; sedimentation[6, 7] rate 5.5 per cent, 14.5 per cent, 43 per cent; Weltmann[8] coagulation band 8. Ten sputum specimens were negative for tubercle bacilli. Cultures of the sputum on Sabouraud's glucose agar resulted in a growth of aspergillus, whose cultural characteristics are as follows: Milk coagulated, then liquefied; broth, large whitish surface colony; Sabouraud's and Krainsky's agar, tough, dull, yellowish-white growth; carrot

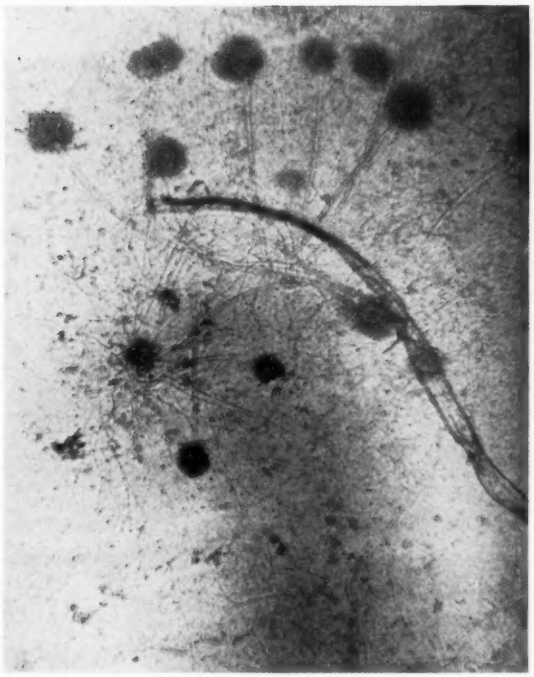

Fig. 3*A*.—Slide culture of the aspergillus showed mycelial strands from which branched stalks bearing conidia clustered about the conidiophore.

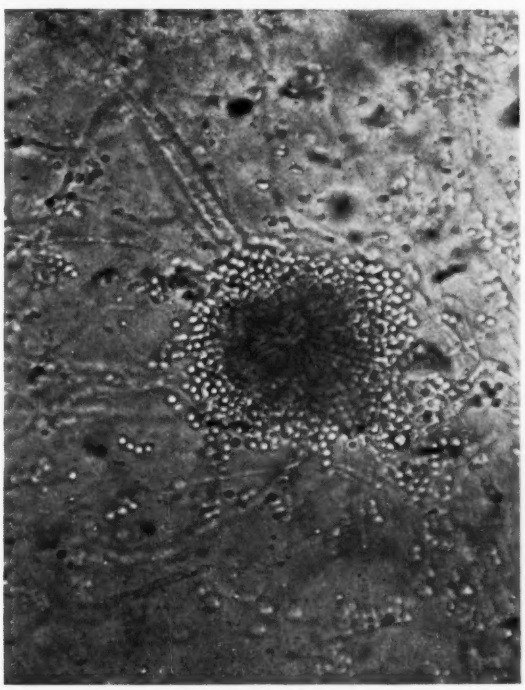

Fig. 3*B*.—Higher magnification of the slide culture revealed the structural arrangement of the spore-bearing stalk.

infusion agar, colonies small, round, and spinose; gelatin stab, white, cottonish surface growth. Acid but no gas was formed in dextrose, saccharose, maltose, and lactose.

Microscopic examination revealed a gram-negative nonacid-fast organism, exhibiting a branching mycelium with a basal stem and a stalk which terminated in a bulbous enlargement or conidiophore around which were clustered numerous conidia (Figs. 3*A* and 3*B*). The conidia ranged in size from one-third to one-half the size of an erythrocyte.

Intravenous injection of one-half of a forty-eight-hour agar slant culture of the aspergillus caused the death of a grown rabbit in three days. Post-mortem examination of the rabbit showed large hemorrhagic areas throughout both lungs, liver, and spleen, with petechial hemorrhages present in the cortex of the kidneys. Cultures from the various organs were planted on Sabouraud's agar, and a pure culture of the organism was recovered from the spleen.

From these findings a diagnosis of pulmonary aspergillosis was made, and the patient was placed upon iodide therapy. Potassium iodide was given orally in increasing doses until 40 drops were given three times daily. The patient's symptoms have disappeared after nine months' treatment and he has gained 45 pounds in weight.

COMMENTS

It has often been emphasized that aspergillosis has a distinct relationship to certain occupations. This is most commonly observed among bird fanciers[10] and pigeon stuffers[11] who feed the birds from their own mouths. This occupation affords a double hazard in the infected birds and the contaminated grain. Other occupations in which there is prolonged exposure to grain dust, such as occurs in farming, milling, threshing, and sponge cleaning afford ample opportunity for infection with this organism.[1, 3] It is likely that often repeated massive dosage is the chief factor in the pathogenesis of this disease. Asymptomatic cases have been found upon routine examination of iron miners in certain localities.

The concomitant occurrence of aspergillosis and other pulmonary diseases has prompted many investigators to advance the theory that aspergillosis does not occur in man unless the local or constitutional resistance is lowered. "Secondary aspergillosis" has been reported to complicate pulmonary tuberculosis, primary carcinoma,[9, 13] and other pulmonary conditions. Lowering of the general resistance by such diseases as uncontrolled diabetes, carcinoma, dysentery, and enteritis predisposes to the infection of the lungs with aspergillosis.

Lapham[4] in 1926 reported ten cases of primary aspergillosis which she divided into two groups, the wet or parenchymatous and the dry or interstitial types. However, the classification most often employed recognizes a superficial and a deep form. In the superficial type aspergillary catarrh and bronchitis are included. The deep type produces necrosis, and caseation of the lung tissue occurs. This organism shows a high[4] and constant virulence for laboratory animals. Large doses of the spores intravenously cause death of the animal in one or two days. The lesions produced by this massive infection showed only hemorrhagic and necrotic areas. Smaller doses cause death of the animal in a week or ten days. In studying these tissues Henrici noted that tubercles resembling those produced by tuberculosis resulted.[5]

The onset of the disease is insidious and may be manifested only by cough, asthmatic attacks, and anorexia. Later the cough is increased and the sputum

becomes tenacious and may be dotted with black specks[14] which are conidia. Night sweats, an evening rise in temperature, loss of weight, and hemoptysis occur as the disease progresses. Massive hemorrhages may be the first symptom that causes the patient to seek medical advice. In the asthmatic type progressive dyspnea is a distressing symptom. Pleurisy may be the most outstanding symptom as the patient relates his history.

Cavitation and destruction of the lung tissue are slow, and the course is protracted. However, when mixed infection of the diseased tissue occurs, the progression of the disease may be much more rapid.

As with other pneumomycoses, there is no characteristic x-ray picture. Involvement of the bases and hilar regions is most common. Limitation of the involvement to these areas is by no means constant.[16] Increased hilar shadows with radially distributed increased markings are said to be one of the earliest pictures presented by this disease. The x-ray may resemble bronchopneumonia in the early stage of resolution.[3]

Physical examination may reveal limited expansion of the chest, most marked on the side with the greater degree of involvement. Coarse and fine râles may be observed upon auscultation over the bases or hilar areas.

Diagnosis of aspergillosis must be made only when all other possible etiologic factors are ruled out. The finding of the aspergillus organism in the sputum is not sufficient evidence for making such a diagnosis. Culturing the organism, the reproduction of the lesion by means of animal inoculation, and the reisolation of the fungus are important factors in clinching the diagnosis. The presence of agglutinins in the patient's serum and positive skin reaction to stock or autogenous vaccines are additional aids in the diagnosis, although their value is questioned by some authorities.

In the treatment of pneumomycoses in general, a multitude of agents have been used. The same is true of the treatment of aspergillosis. The time-honored remedy employed in the condition is potassium iodide;[12] other medicaments often used are the inhalation of ethyl iodide,[1] neoarsphenamine intravenously, intramuscular bismuth, sodium iodide intravenously,[15] and the roentgen ray. Of course, the presence of concomitant pulmonary tuberculosis contraindicates the use of iodides. It is easily seen that there is no "specific" drug.

SUMMARY

1. Pneumomycosis should be considered in any patient with pulmonary symptoms.

2. A case of pulmonary aspergillosis was presented with a discussion of the mode of infection, pathology, symptomatology, x-ray picture, diagnosis, and treatment.

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THE PERCUTANEOUS POTENCY OF PROGESTERONE*

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IT HAS been known for some time that estrogens and androgens are absorbed through the skin of the experimental animal and produce systemic effects.[1-8] Various workers have made clinical observations demonstrating that the same effect is obtained in the human being.[1, 9-12] Unfortunately, both estrogens[1, 2] and androgens[7, 8] are much less potent when given percutaneously in an ointment than when given parenterally. Investigations on the percutaneous effectiveness of progesterone are limited to a statement by Zondek that in the rabbit it is less effective by this route than by subcutaneous injection.[2]

After parenteral administration progesterone is relatively rapidly absorbed, metabolized, and excreted. It resembles estrone or testosterone in this respect. The estrogens and androgens, however, may be given parenterally in the esterified forms (e.g., estradiol dipropionate and testosterone propionate) which are relatively slowly absorbed and have a prolonged effect. Thus the patient may be given adequate therapy with treatment at infrequent intervals. Progesterone, however, has no active esterified form; consequently, the patient should be treated frequently, probably every day, to obtain the best clinical response. The indications, if any, for progesterone therapy and the inadequacy of the dosages commonly employed are not pertinent to this discussion.

Since percutaneous application of progesterone would be a convenient method for daily treatment, it was decided to investigate the percutaneous potency of progesterone. A quantitative procedure such as this was not

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feasible in the human being; accordingly, the rabbit was used. We realize, of course, that skin absorption of progesterone is not necessarily the same in the rabbit as in the human being. Therefore, the results obtained in the rabbit can only be considered as indicative of what might happen in the human being.

PROCEDURE

Thirty-seven virgin immature rabbits were used in this study. All were primed with 0.002 mg. estrone on the first and third days of the experiment. Five animals were given no further treatment and served as controls. The remaining animals were given progesterone* daily, starting on the fifth day. All animals were killed on the tenth day. The uteri were dissected out, weighed, fixed in Bouin's fluid, and prepared for microscopic study. The progesterone-treated animals were divided into eight groups (Table I). One group was treated parenterally, and the remainder were treated percutaneously. The substance for parenteral administration was applied to an area on the back from which the hair was clipped.

TABLE I

TREATMENT (DAILY DOSE)	NO. OF ANIMALS	AVERAGE BODY WEIGHT (GM.)	AVERAGE UTERINE WEIGHT (GM.)	McPHAIL RATING (AVERAGE)
Control (primed)	5	667	0.364	0
Subcutaneously 0.1 mg. (oil)	5	683	0.862	2.6
Percutaneously 0.1 mg. (oil)	2	630	0.383	1.0
Percutaneously 0.3 mg. (oil)	4	654	0.456	1.25
Percutaneously 0.6 mg. (oil)	4	694	0.765	2.37
Percutaneously 0.1 mg. (alcohol)	5	684	0.561	1.4
Percutaneously 0.2 mg. (alcohol)	5	763	0.590	1.6
Percutaneously 0.4 mg. (alcohol)	5	549	0.995	3.0
Percutaneously 0.6 mg. (alcohol)	2	653	1.697	4.0

In all cases the progesterone was given in 0.1 c.c. of solvent. Alcohol was used as well as oil for percutaneous application, since it has been demonstrated that both estrogens and androgens are more potent when applied percutaneously in this solvent.[2, 6, 7]

The progestational responses of the uteri were graded according to the histologic method of McPhail.[13]

RESULTS

The mean uterine weights and progestational responses, as well as the treatment details for each group, are presented in Table I.

The number of animals in each group is adequate for an estimation only of the potency of progesterone when given by different methods. It is obvious, however, that in an oil solvent progesterone is not very effective by the percutaneous route. With 0.1 mg. the uteri were not significantly larger than those of the castrate controls, and histologically they showed only slight stimulation. The effect was only slightly greater with 0.3 mg., and even 0.6 mg. produced uteri which were smaller and had a lower histologic rating than 0.1 mg. parenterally.

*The progesterone used in this study was furnished by Schering Corporation.

The alcohol solvent definitely increased the effectiveness of the percutaneous progesterone. However, even in this solvent the percutaneous route was less effective than the parenteral, 0.1 and 0.2 mg. both produced smaller uteri and lower histologic ratings than did 0.1 mg. parenterally. However, 0.4 mg. percutaneously produced a somewhat greater effect than 0.1 mg. parenterally.

DISCUSSION AND SUMMARY

These data indicate that progesterone is less effective by the percutaneous route than by the parenteral method. With oil as the solvent it is less than one-sixth as potent. With alcohol as the solvent it is less than one-half, but more than one-fourth, as potent percutaneously as parenterally.

Any clinical application of these data must rest on the assumption that skin absorption of progesterone is the same in man as in the rabbit. The validity of this assumption can only be tested by clinical experience. Assuming, however, that the skin of the human being would react in a manner similar to that of the rabbit, it is obvious that very large doses of progesterone would have to be given to obtain a satisfactory clinical response.

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THE DETERMINATION OF THE DOSAGE-MORTALITY RATIO OF PENTOTHAL SODIUM WITH SULFANILAMIDE*

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WITH the increasing use of sulfanilamide in the fields of medicine and surgery, the anesthetist is frequently called upon to administer an intravenous anesthetic, particularly pentothal sodium (sodium ethyl 1-methyl butyl thiobarbiturate), to a patient who has been receiving sulfanilamide (para-aminobenzenesulfonamide) or one of its derivatives. Adriani[1] has suggested that the sulfanilamide-barbiturate sequence may be unwise in human therapy.

In his experiments on rats previously treated with 0.5 to 1.0 Gm. per kilogram of sulfanilamide, a dose which Nelson[2] describes as fatal for rabbits, susceptibility to anesthetic doses of the barbiturates was increased. The thiobarbiturates were the worst offenders in this respect.

The present study was undertaken with the purpose of determining whether the toxicity of pentothal sodium is increased by previous administration of sulfanilamide in therapeutic doses (0.15 Gm. per kilogram per day). The above dose agrees fairly well with that recommended by Long and Bliss,[3] who used 0.11+ Gm. of sulfanilamide per kilogram for human therapy.

The animals used were white Wistar strain rats, between the ages of six weeks and three months, weighing approximately 150 Gm. Sulfanilamide was prepared by dissolving the crystals in sterile distilled water to make a 1 per cent solution. Intraperitoneal injections of 0.15 Gm. of sulfanilamide per kilogram of body weight were then made daily for seven days. On the eighth day pentothal sodium (2.5 per cent solution) was given intraperitoneally to eight groups of 10 rats each, each group receiving a different dose. The doses ranged from 50 to 110 mg. per kilogram. As a control eleven groups of 10 rats each were given 40 to 140 mg. per kilogram of pentothal sodium, having had no previous treatment with sulfanilamide.

Computations of the L. $D._{50}$ were carried out according to the method of Bliss,[3] using the equation $\log \text{L. } D._{50} = \bar{x} + s(5 - \bar{y})$ for the determination of the logarithm of the median lethal dose, and the equation $V(\log \text{L. } D._{50}) = s^2\left(\frac{(5-\bar{y})^2}{(wxy)} + \frac{1}{S(w)}\right)$ for the standard error of deviation.

From the data in Table I the log of L. $D._{50}$ for pentothal sodium alone, and for pentothal sodium with sulfanilamide has been computed, with their standard

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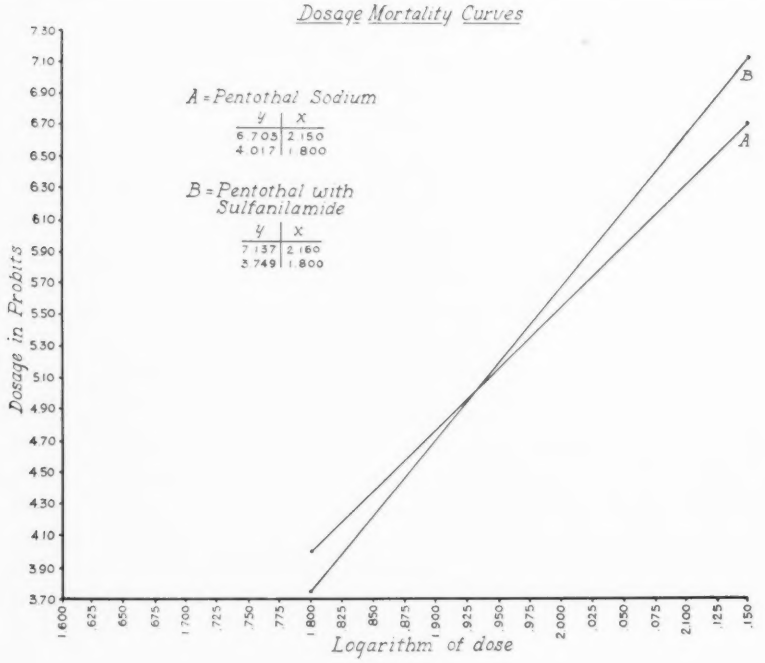
errors. Graph 1 shows the computed curves for pentothal sodium and pentothal sodium with sulfanilamide.

TABLE I

TOXICITY OF PENTOTHAL SODIUM AND OF PENTOTHAL SODIUM SUBSEQUENT TO SULFANILAMIDE THERAPY

NO.	PENTOTHAL SODIUM		PENTOTHAL SODIUM WITH SULFANILAMIDE (15 MG.)	
	DOSE IN MG.	NUMBER OF RATS DEAD/TOTAL	DOSE IN MG.	NUMBER OF RATS DEAD/TOTAL
1	40	0/10		
2	50	1/10	50	0/10
3	55	1/10		
4	60	1/10	60	1/10
5	65	2/10		
6	70	3/10	70	2/10
7	75		75	3/10
8	80	3/10	80	4/10
9	90	5/10	90	6/10
10	100	6/10	100	7/10
11	110	9/10	110	9/10
12	140	10/10		
	$L.D._{50}$	84.85 ± 4.00		85.2 ± 6.56



Graph 1.

RESULTS

By the above method the median lethal dose (L. $D._{50}$) for pentothal sodium was computed to be 84.85 ± 4.00 mg. per kilogram. This result is in fair agreement with that of Pratt and his co-workers,[5] who found the M. L. D. for pentothal sodium in rats to be 80 mg. per kilogram, although they do not state how they arrived at this figure. The median lethal dose (L. $D._{50}$) for pentothal

sodium in rats which had previously received therapeutic doses of sulfanilamide was calculated to be 85.21 ± 6.56 mg. per kilogram. By comparison of these median lethal doses ($L. D._{50}$) there is no significant difference in the toxicity of pentothal when given to rats which have received therapeutic doses of sulfanilamide.

CONCLUSION

These data justify the conclusion that sulfanilamide in therapeutic doses does not increase the toxicity of pentothal sodium given subsequently to white rats, does not render the animals more susceptible to the effects of pentothal sodium, nor do the two drugs exert synergistic action, at least of a harmful nature, with this dosage.

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IMMUNITY STUDIES OF CRYPTOCOCCUS HOMINIS (TORULA HISTOLYTICA) IN MICE*

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A REVIEW of published reports (see references†) demonstrates that a mild form of hypersensitivity, usually revealed by skin reactions, occurs in patients infected with torula. Tests for serum antibodies are inconclusive. This study was undertaken to determine evidence of immunity against torula in a highly susceptible animal, such as the white mouse.

MATERIALS AND METHODS

The *Cryptococcus hominis* strain used for the study was isolated in 1935 from a patient with torular meningitis. Growth on glucose agar was abundant in forty-eight hours at 37° C. Cultures from slants of this medium were suspended in normal saline, and the concentrations were estimated with a hemocytometer. Other methods for standardization of suspension were unsatisfactory. The suspensions were heated for fifteen minutes at 100° C. The torula were killed by two to four minutes' exposure to this temperature. Aqueous

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extracts were obtained by Berkefeld filtration of old cultures (two and a half months) in peptone broth and synthetic medium.* A concentrate of the synthetic medium was prepared by precipitation with four parts of acetone and reprecipitation from normal saline solution. The final volume was one-tenth that of the original filtrate. Serologic tests were made with torula treated by the Stoddard and Cutler method. To a flask containing a saline suspension of torula, 0.1N hydrochloric acid was added to a pH of 4. After the mixture digested for forty-eight hours at 10° C., the organisms were removed by centrifugation and washed free of acid. The torula were used as the agglutinogen and the neutralized acid extract was used as the precipitinogen for the demonstration of serum antibodies.

DETERMINATION OF THE MINIMUM LETHAL DOSE

Each of 18 mice received 300,000,000 live torula intraperitoneally. This dose was established by preliminary experiments. All the mice died within twenty-five days; 40 per cent died in eleven days; 50 per cent in twelve days; 70 per cent in fourteen days. Using twelve days arbitrarily as the time limit, and 50 per cent as the number of mice killed with a dose of 300,000,000 torula, an arbitrary M.L.D. was obtained with a range of error of 11 per cent.

IMMUNITY FROM INJECTIONS OF DEAD TORULA

Twenty-three mice were immunized by twelve injections of 30,000,000 heat-killed torula at three-day intervals. The mice were then infected with live torula as was an equal number of control mice. All nonimmunized mice were dead in sixteen days, and the immunized mice in seventeen days. Twelve control mice and six immune mice were dead after eight days. On the tenth day the ratio was 18:10; on the twelfth day, 22:15. In the immunized group the death rate from the seventh to twelfth days was approximately 50 per cent that of the nonimmunized mice.

The gross pathology at post mortem did not differentiate the immune mice from the control mice. In the histologic examination of the immune mice, the lungs varied from complete susceptibility, represented by a massive hemorrhagic pneumonia, to a moderate degree of immunity, represented by patches of proliferated cells and thickening of the alveolar walls by hyperplasia of the septal cells. Immune changes observed in the brain were: fewer and smaller cysts, fewer torula, and single torula without cyst formation. Some brains had plaques of proliferated cells where the pial vessels course through the cortex. Occasionally there was proliferation of the endothelium of the choroid plexuses.

In experiments performed with rats, pairs of immunized and control rats were killed at seven-day intervals following the infecting dose. Thus the evolution of the lesions, as well as the character of the changes, could be observed. The histologic immune process early consisted of cellular proliferation of the septal cells of the lung, reticulo-endothelial cells of the spleen and the liver, and connective tissue growths in the omentum forming irregular focal regions of compact cellular tissue. As these changes progressed, foreign body giant

*Distilled water with 6 per cent ammonium lactate, 0.2 per cent dipotassium phosphate, and 0.5 per cent sodium chloride.

cells with torula appeared. Later, the fibrous tissue elements became more prominent, and loosely woven nodules of fibrous tissue in whorls with epithelioid cells formed. Langhans giant cells were numerous and in combination with the nodules resembled tuberculous foci. The immunity expressed itself mainly as a hastening of the stages of cellular proliferation with subsequent scar tissue reaction and disappearance of the torula.

IMMUNITY FROM INJECTION OF BROTH FILTRATE

The immunity obtained in these animals was comparable to that of the previous group, but less marked. Attempts to reveal soluble toxins gave negative results.

SENSITIZATION TO TORULA

Mice were infected with torula, each mouse receiving 25,000,000 organisms. After twelve, sixteen, and twenty days, tests for hypersensitivity by intradermal injections on the shaven abdomens gave no reactions. The test solutions were broth filtrate, filtrate of the synthetic medium, and the concentrate of the latter filtrate. Intratesticular injections also were reactionless. Skin tests on an immunized rabbit were likewise negative. Accordingly, either the time required for the development of sensitization was long or these animals were not susceptible to such a response to this strain of torula.

DETERMINATION OF HUMORAL IMMUNITY

Six intravenous injections of heat-killed torula were made in a rabbit at three-day intervals. One cubic centimeter of dilutions of immune and control sera was mixed with 1 c.c. of a suspension of torula prepared as described, and placed in a water bath at 37° C. The suspensions of torula were stable for four hours. At the end of one hour the agglutination titer was 1:180. After five hours it was 1:280, with 1:60 in the controls. Agglutination tests with three different control serums compared with saline controls proved there was no nonspecific agglutination.

SUMMARY

1. Injections of torula suspension killed by heat prolonged life and modified the histologic structure of the lesions in mice subsequently infected with torula.

2. No exotoxin or cutaneous hypersensitivity was demonstrated with this strain of torula.

3. Agglutinins were demonstrated in the serum of an immunized rabbit to a titer of 1:180.

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THE EFFECT OF SPLENIC EXTRACTS FROM CASES OF ESSENTIAL THROMBOCYTOPENIC PURPURA ON THE PLATELETS AND HEMATOPOIETIC ORGANS OF RABBITS*

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THE relation of the spleen to the production of essential thrombocytopenic purpura has for some time been a subject of much dispute among investigators. In 1915 Frank[1] suggested that the spleen was responsible for the formation of an agent which exerts a destructive action upon the megakaryocytes of the bone marrow. The assumption that this toxin is capable of involving the bone marrow as a whole was proposed by Minot[8] in 1917. Kaznelson,[5] who first removed the spleen in a case of essential thrombocytopenic purpura, believed that the thrombocyte reduction in the peripheral blood was brought about by an increased thrombolytic activity of the spleen. Torrioli and Puddu[13] showed injury of the megakaryocytes in bone marrow cultures after the application of concentrated doses of splenic extracts from cases of essential thrombocytopenic purpura.

The recent investigations of Troland and Lee[15, 16] demonstrate a marked depression of the circulating blood platelets in rabbits after intravenous injection of acetone extracts of splenic tissue taken from three patients operated on for essential thrombocytopenic purpura. In September, 1939, Pohle and Meyer[9] reported that acetone extracts of the spleen from three patients with essential thrombocytopenic purpura produced no effect on the platelet levels of injected rabbits. Major and Weber[7] injected extracts prepared from the spleens of two

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patients with essential thrombocytopenic purpura. The splenic extract from one patient produced an increase in the blood platelet count, while the extract from the second patient caused a moderate fall in the platelet level. Tocantins[12] was unable to demonstrate the presence of platelet-destroying substances in the spleen and urine of three patients with thrombocytopenic purpura. Following the injection of 45 c.c. of an acetone extract of a thrombocytopenic spleen into a rabbit, Hobson and Witts[3] reported a depression in the platelet count from 525,000 to 109,000 in thirty hours. Rose and Boyer[10] record studies which are confirmatory of the work of Troland and Lee.[15, 16] Because of the bearing of these experiments on the mechanism of the production of essential thrombocytopenic purpura, it seemed important to repeat them and to include in the study changes in the bone marrow and hematopoietic organs.

MATERIAL AND METHODS

Extracts were prepared from splenic tissue obtained from five patients with essential thrombocytopenic purpura and from six other control patients with splenic enlargement. Sufficient clinical and hematologic data are presented to establish the accuracy of the diagnosis of the cases.

CASE 1.—Patient With Essential Thrombocytopenic Purpura:

D. T., a white girl, aged 14 years, was admitted to the Illinois Research and Educational Hospitals on March 10, 1938, complaining of frequent attacks of epistaxis and excessive menstrual bleeding. Bleeding from the nose was first noticed in October, 1937, at which time she experienced a nosebleed which persisted for two days. Menstrual period in November, 1937, continued for nine days, and in December, for fourteen days.

Physical examination was essentially negative, except for numerous petechiae, especially over both lower extremities. The spleen was not palpable. Blood studies on entrance showed platelets 48,040, erythrocytes 3,680,000, hemoglobin 8.5 Gm., and a definite megakaryocytic increase of the sternal bone marrow. On March 19 the bleeding time was three minutes and the coagulation time (Howell's method) was ten minutes. There was no clot retraction at the end of twenty-four hours and the tourniquet test was positive. Blood studies on March 25 showed platelets 61,200, erythrocytes 2,930,000, hemoglobin 7.25 Gm., and megakaryocytic hyperplasia of the sternal bone marrow. Splenectomy was performed on April 11, 1938. The spleen weighed 157 Gm. Twenty-four hours after operation the platelet count was 72,300. The platelets increased progressively, and on April 18 (seven days after splenectomy) they numbered 386,400. Following splenectomy there has been no bleeding at any time.

CASE 2.—Patient With Essential Thrombocytopenic Purpura:

R. V., a white woman, aged 22 years, admitted to the Illinois Research and Educational Hospitals on June 24, 1938, had frequent attacks of epistaxis. Nasal hemorrhages commenced about Feb. 15, 1938, and usually persisted for about one-half hour. She continued to have two or three hemorrhages weekly up until June 1, when the attacks became more frequent and prolonged. Menstrual history was normal until June 12, 1938, and continued until July 1, with a marked increase in the bleeding. Physical examination revealed a moderate pallor of the skin and numerous bright red petechiae over chest, abdomen, back, and extremities. The spleen was palpable and the tourniquet test was strongly positive. Blood studies on entrance showed platelets 24,400, erythrocytes 2,400,000, hemoglobin 6.3 Gm., and marked increase of the megakaryocytes on sternal bone marrow examination. The coagulation time was five minutes and the bleeding time was eight minutes. There was no clot retraction at the end of twenty-four hours. Splenectomy was performed on June 30. The spleen weighed 270 Gm. Five hours postoperatively there were 136,200 platelets with many large forms. Twenty-four hours after splenectomy platelets numbered 185,400, and on July 11 (eleven days after surgery) the platelet count was 473,000.

CASE 3.—Patient With Essential Thrombocytopenic Purpura:

F. R., a white woman, aged 21 years, first entered the Illinois Research and Educational Hospitals on Dec. 5, 1938, complaining of frequent attacks of epistaxis, bleeding from the gums, vagina, and rectum. She was in good health until the latter part of October, 1938, when she first noted a small amount of bright red blood after defecation. Shortly afterwards she began to have frequent attacks of epistaxis. The menstrual period during the first week of November was very profuse and continued for eight days. Physical examination revealed numerous petechiae distributed over both lower extremities. The spleen was palpable, and the tourniquet test was markedly positive. Blood studies on entrance showed a platelet count of 29,740, erythrocytes 4,970,000, hemoglobin 13.6 Gm., and a megakaryocytic hyperplasia of the sternal bone marrow. The coagulation time was eight minutes and the bleeding time was ten minutes. There was no clot retraction at the end of thirty hours. Splenectomy was performed on Dec. 14, 1938. The spleen weighed 132 Gm. Twenty-four hours after operation the platelet count was 171,400, and on December 19 the platelets numbered 314,700. Bleeding subsided completely following the splenectomy.

CASE 4.—Patient With Essential Thrombocytopenic Purpura:

P. H., a white boy, aged 15 years, was admitted to the Illinois Research and Educational Hospitals on Sept. 12, 1939, complaining of epistaxis. Bleeding from the nose was first experienced in June, 1939, and was followed in a short time by persistent oozing of blood from the gums. Physical examination was essentially negative, except for numerous petechiae over the posterior aspect of the neck, cubital region, and chest. Blood studies on entrance showed platelets 40,000, erythrocytes 4,490,000, hemoglobin 13.0 Gm., and a definite megakaryocytic increase of the sternal bone marrow. The bleeding time was twenty minutes and the coagulation time was nine minutes. There was no clot retraction at the end of twenty-four hours. The patient remained in the hospital under observation for several months. During this time he had many severe attacks of epistaxis. Platelet counts as low as 11,000 were obtained. Splenectomy was performed on Dec. 21, 1939. The spleen weighed 290 Gm. Six hours after removal of the spleen the platelets numbered 76,000, and eight days postoperatively a count of 980,000 was obtained.

CASE 5.—Patient With Essential Thrombocytopenic Purpura:

B. P., a white girl, aged 17 years, was admitted to the Illinois Research and Educational Hospitals on July 26, 1940. She had severe epistaxis, bleeding gums, purpuric spots, and bruised easily since December, 1938. At the time of entrance she was pregnant approximately seven months. Physical examination revealed red, spongy gums. The liver was palpable and the tip of the spleen could be felt. The bleeding was controlled, and the patient was allowed to go into labor spontaneously on Sept. 29, 1940. The puerperium was afebrile and uneventful. Blood studies on admittance to the hospital showed platelets 100,000, erythrocytes 3,120,000, hemoglobin 9.8 Gm., and a myeloid erythroid and megakaryocytic hyperplasia of the sternal bone marrow. The bleeding time was twelve minutes, the coagulation time was nine minutes, and the beginning clot retraction occurred at the end of one and one-half hours. She was prepared for splenectomy by the administration of blood, and on Oct. 23, 1940, the spleen was removed. Twenty-three days postoperatively blood studies revealed platelets 200,000, erythrocytes 5,910,000, and hemoglobin 17.5 Gm. Since splenectomy there has been no bleeding from the nose or gums.

Splenic tissue obtained from six control cases was used in the preparation of extracts. They included a normal spleen and cases of congenital hemolytic anemia, splenic vein thrombosis, chronic lymphatic leucemia, aplastic anemia, and Banti's syndrome.

The method of extraction used in this investigation has been described in detail by Troland and Lee.[15, 16] Suffice it to say that the macerated splenic tissue was placed in acetone. After a variable length of time the acetone was filtered off and then distilled. The sediment was dissolved in 100 c.c. of distilled water

and filtered. Extracts were stored in the refrigerator and injected within forty-eight hours after preparation. The type of acetone (commercial or reagent) used in the extraction seemed to have no influence on the results obtained. Table I contains the extracts prepared, type of acetone used in the extraction, weight of the splenic tissue, grams of the splenic tissue in a 20 c.c. dose of the splenic extract, and the length of time the tissue was allowed to remain in the acetone.

TABLE I

SPLENIC EXTRACTS

SPLENIC EXTRACTS	TYPE OF ACETONE	WEIGHT OF SPLENIC TISSUE IN GRAMS	TIME OF EXTRACTION IN ACETONE IN DAYS	GRAMS OF SPLENIC TISSUE IN 20 C.C. SPLENIC EXTRACT	GRAMS OF SPLENIC TISSUE IN 10 C.C. SPLENIC EXTRACT
E. 1. Thrombocytopenic purpura	Commercial	130	253	26.0	13.0
E. 2. Thrombocytopenic purpura	Reagent	140	182	28.0	14.0
E. 3. Thrombocytopenic purpura	Reagent	109	35	21.8	10.9
E. 4. Thrombocytopenic purpura	Reagent	82	274	16.4	8.2
E. 5. Thrombocytopenic purpura	Reagent	203	30	40.6	20.3
E. 6. Normal control	Reagent	125	19	25.0	12.5
E. 7. Splenic vein thrombosis	Reagent	114	291	22.8	11.4
E. 8. Congenital hemolytic anemia	Reagent	285	51	57.0	28.5
E. 9. Chronic lymphatic leucemia	Reagent	123	91	24.6	12.3
E. 10. Aplastic anemia	Reagent	127	61	25.4	12.7
E. 11. Banti's syndrome	Reagent	250	18	50.0	25.0

In the present study the number of blood platelets was determined by the direct method. In some instances the indirect blood smear method was used to check the results obtained by the direct counts. The diluting fluid used consists of 0.6 Gm. sodium chloride, 0.2 Gm. sodium citrate, and 0.04 Gm. brilliant cresyl blue made up to 100 c.c. with distilled water. Blood was obtained from a stab wound of the marginal vein of the rabbit's ear. The blood was drawn to the 0.5 mark in a standardized erythrocyte pipette and diluted to the 101 mark with the platelet diluting fluid. After shaking for two minutes a certified counting chamber with improved Neubauer ruling was filled and allowed to stand for ten minutes. Counts were made with a high, dry (4 mm.) objective and a 12.5× ocular.

Normal rabbits, approximately 2 kg. in weight, were employed as the experimental animals. A new rabbit given no food for twelve hours previously was used for each experiment. Several control platelet counts were made prior to the injection of the splenic extracts. After administration of the extract, platelet counts were repeated every two to four hours for a period of twenty-four to forty-eight hours.

Bone marrow sections and bone marrow imprints, together with sections of the liver, lung, and spleen were obtained from seven animals. After injection of 20 c.c. of the extracts prepared from the essential thrombocytopenic purpura specimens, the rabbits were sacrificed at the maximal platelet count depression. Following the injection of a control extract (E. 7), the rabbit was sacrificed at the time interval required to produce maximal platelet drop with the essential thrombocytopenic purpura extracts. Specimens of the bone marrow, liver, lung, and spleen were also obtained from a normal untreated rabbit. Pieces of tissue were fixed in a solution of formaldehyde and in Zenker's fluid. After preparation of the sections they were stained by hematoxylin and eosin and Giemsa's stains.

On examination of the bone marrow sections the number of megakaryocytes per 1,500 nucleated bone marrow cells was determined. The bone marrow imprints were prepared by touching the cut end of the sternum to a glass slide. Each slide contained eight such touch smears of approximately equal size. In enumerating the megakaryocytes on the bone marrow imprints all megakaryocytes exclusive of the central portion of the imprint were counted. The central portion of the imprints were not included in the counts because of the deep stain which they assumed, preventing accurate cellular differentiation. The megakaryocyte counts of the imprints were determined by taking the average of eight imprints.

EXPERIMENTAL OBSERVATIONS

Splenic extracts prepared from the essential thrombocytopenic purpura specimens, Cases 1, 2, 3, 4, 5, will be designated as extracts (E) 1, 2, 3, 4, 5, respectively. After injection of 20 c.c. of E. 1, a depression in the platelet count was noted from the preliminary count of 500,000 to 325,000 within eight hours. Twelve hours after injection the platelet count was 450,000, and within twenty-four hours it had reached the preinjection level.

Injection of 20 c.c. of E. 2 produced practically the same result as that of E. 1. A depression in the platelets from the preinjection level of 492,000 to 315,000 was obtained within eight hours. Twenty hours later the platelet count was 440,000, and within twenty-four hours it reached the level determined prior to injection of the extract.

E. 3 differed from the previously mentioned extracts in that the maximal platelet depression was not reached until sixteen hours had elapsed. After injection of 20 c.c. of E. 3 the platelet count fell from a preinjection level of 485,000 to 315,000. The platelets returned to the preinjection level within twenty-four hours.

Following the injection of 20 c.c. of E. 4, a depression in the platelet count was noted from the preliminary count of 525,000 to 455,000 within twelve hours. At the end of twenty-four hours a count of 495,000 was obtained. The experiment was repeated, using 40 c.c. of E. 4. This dosage produced a drop in the platelet count from the preliminary level of 495,000 to 350,000 within twenty-four hours.

Injection of 20 c.c. of E. 5 caused a depression in the platelet count from a preliminary level of 500,000 to 405,000 within four hours. At the end of twenty-four hours the platelets numbered 485,000.

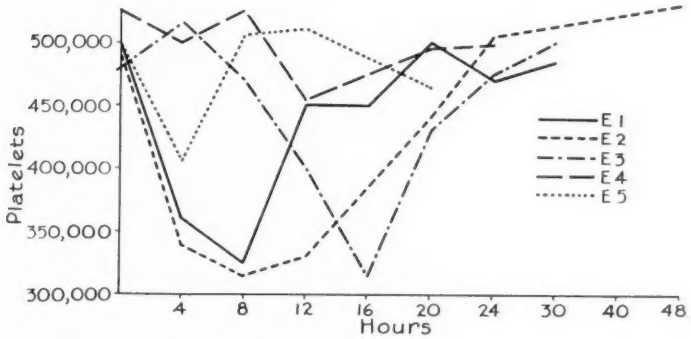

Fig. 1.—Platelet level following injection of 20 c.c. of thrombocytopenic extracts.

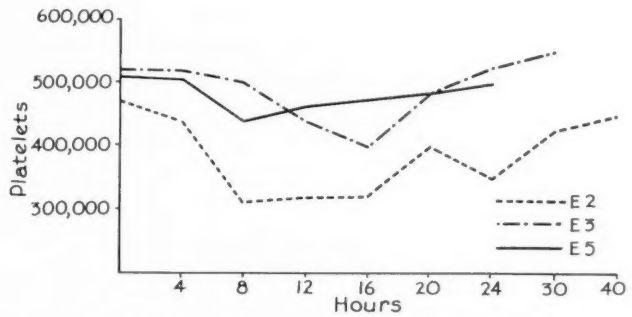

Fig. 2.—Platelet level following injection of 10 c.c. of thrombocytopenic extracts.

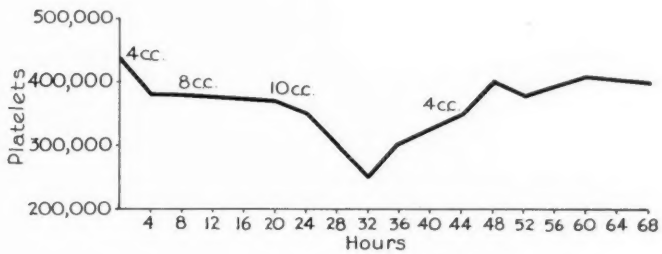

Fig. 3.—Determination of minimal effective dose.

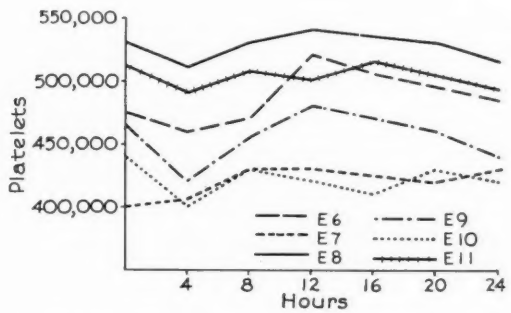

Fig. 4.—Platelet level following injection of control extracts.

The experiments were repeated using 10 c.c. of the splenic extracts, E. 2, E. 3, and E. 5. After the injection of E. 2 a depression of the platelet level resulted that was essentially the same as that produced by 20 c.c. of the extract.

Ten cubic centimeters of E. 3 produced a less marked depression of the platelets. The platelet count prior to injection was estimated at 520,000; sixteen hours later it was 400,000; and within twenty-four hours it had attained the preinjection level.

The injection of 10 c.c. of E. 5 was followed by a maximal platelet depression of 75,000 within eight hours.

In an attempt to determine the minimal effective dose of the extract, 4 c.c. of E. 2 were injected intravenously. A depression of 55,000 in the platelet count was obtained within eight hours. At this time 8 c.c. of the extract was injected. Twelve hours later the count dropped an additional 10,000 platelets. Then 10 c.c. of the extract was injected, and it was noted that a total maximal platelet depression of 185,000 was reached twelve hours after the injection of 10 c.c. of the extract.

Experiments using the control specimens were carried out with the same procedure employed in the cases of essential thrombocytopenic purpura. Twenty cubic centimeters of the extract (E. 6) prepared from the normal spleen were injected. A depression in the platelet count from 475,000 to 460,000 in four hours was noted. There was a return to the preinjection level within twelve hours. Other control specimens produced the following results:

After the injection of 20 c.c. of E. 7 (splenic vein thrombosis) no depression was noted. Twenty-four hours later the platelet count exceeded the initial count by 28,000. Twenty cubic centimeters of E. 8 (congenital hemolytic anemia) caused a drop of 22,000 platelets within four hours with a return to the preinjection level within eight hours. Following the injection of E. 9 (chronic lymphatic leucemia) a depression of 45,000 platelets within four hours was observed, with a return to the preliminary count within twelve hours. Ten cubic centimeters of E. 10 (aplastic anemia) produced a depression of 40,000 platelets within four hours. After the injection of 20 c.c. of E. 11 (Banti's syndrome) a drop of 22,500 platelets within four hours was noted.

Examination of the bone marrow imprints and sections revealed little, if any, change in the number of megakaryocytes of the rabbits injected with the extracts of thrombocytopenic purpura as compared with the normal and control rabbits.

The only deviation from the constant findings was seen in the case of E. 1. In this case a reduced number of megakaryocytes was found. The megakaryocytes showed no outstanding changes in the cells, such as degeneration, pyknotic nuclei, or vacuolation of the cytoplasm. Sections of the spleen, liver, and lung were studied and failed to show any significant pathologic changes. Particular attention was directed to the examination of the lung sections to determine the presence of megakaryocytes, as reported by Howell and Donahue.[4]

DISCUSSION

It is well established that some relationship exists between the spleen and blood platelets. This is evidenced by the marked and rapid rise in the platelet

count following splenectomy on patients with essential thrombocytopenic purpura, a finding substantiated in all of our cases. The number of circulating blood platelets in the peripheral blood is dependent upon a normal maturation process in the bone marrow. Wright's[17] original finding that the megakaryocytes are the only source of true platelets has been repeatedly confirmed by many investigators. Frank,[2] Limarzi and Schleicher,[6] and Seeliger,[11] have noted in essential thrombocytopenic purpura an increase in the megakaryocytes of the bone marrow associated at times with immature forms. This suggests a maturation arrest of the megakaryocytic tissue and thus speaks for a lack of platelet formation rather than an increased destruction of these elements.

TABLE II

MEGAKARYOCYTE COUNTS

SPLENIC EXTRACTS	BONE MARROW IMPRINTS	BONE MARROW SECTIONS
E. 1	8.1	8
E. 2	14.3	9
E. 3	14.7	12
E. 4	13.8	10
E. 5	11.2	9
Control (Splenic vein thrombosis)	11.7	11
Bone marrow of a normal sacrificed rabbit	12.5	10

Results described elsewhere in this report are not fully in accord with those of Troland and Lee.[15, 16] These workers report a depression of the platelet count to the level commonly seen in persons with essential thrombocytopenic purpura. For the most part, the preparation of the splenic extracts, weight of the splenic tissue, and the method of counting platelets in this study was similar to that employed by Troland and Lee.

It has been demonstrated that the five extracts prepared from the patients with essential thrombocytopenic purpura, when injected intravenously, produce a moderate depression of the circulating blood platelets. This reduction ranges from 70,000 to 177,000 blood platelets, and is very transient in duration. In practically every instance platelet counts returned to the preinjection level within twenty-four hours. Torrioli and Pusic[14] showed that large doses of an aqueous extract of a normal spleen caused a considerable reduction in the circulating blood platelets of rabbits when injected intravenously. An acetone extract of a normal spleen in this series produced no significant depression in the blood platelet count.

Because of the megakaryocytic hyperplasia found in essential thrombocytopenic purpura, bone marrow sections and imprints were obtained at the point of maximal depression of the blood platelets to determine the relationship of the thrombopenia to the megakaryocytes. No disturbances in the number or cell structure of the megakaryocytes were detected.

Due to the short-lived depression of the circulating blood platelets one may speculate that the extract contains a substance which interferes with the rate of formation of the platelets from the megakaryocytes. It is, of course, possible that if these injections were continued for some time an effect on the megakaryocytes would result.

CONCLUSIONS

1. The intravenous injection of acetone splenic extracts of cases of essential thrombocytopenic purpura produces a transient significant depression of the platelet count in rabbits.

2. The splenic extracts exerted no effect upon the number or cell structure of the megakaryocytes of the bone marrow.

3. Specimens of the spleen, liver, and lungs of rabbits obtained after the injection of thrombocytopenic splenic extracts showed no distinctive pathologic changes.

4. The intravenous injection of acetone splenic extracts from six control cases produced no significant change in the level of the platelet count in rabbits.

I wish to express my gratitude to Dr. Robert W. Keeton and Dr. Louis R. Limarzi for their invaluable advice and criticism.

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THE RELIEF OF MUSCULAR WEAKNESS BY PYRIDOXINE HYDROCHLORIDE*

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THE fraction of the vitamin B complex known as B_6 has recently been synthesized. It is chemically described as 2-methyl-3-hydroxy-4, 5-di-(hydroxymethyl)-pyridine.[1, 2] The Council on Pharmacy and Chemistry of the American Medical Association has accepted pyridoxine as the official name of this vitamin. The hydrochloride is to be known as pyridoxine hydrochloride.[3] A deficiency of this substance in rats results in impaired growth and a characteristic dermatitis.[4] Little is known concerning its physiologic activity, but there is some evidence that it is concerned with storage of fat[5] and with the utilization of unsaturated fatty acids.[6]

Our interest was aroused by the early report of Spies and others[7] on the spectacular influence of pyridoxine in relieving the muscular weakness of pellagrins. He reported that pellagrins who had been relieved as regards most signs and symptoms of the disease by vigorous treatment with nicotinic acid, thiamin chloride, and riboflavin, nevertheless still manifested extreme muscular weakness and fatigue. At this time the intravenous administration of 50 mg. of pyridoxine enabled patients (who still complained of extreme nervousness, insomnia, irritability, abdominal pain, weakness, and difficulty in walking) to experience dramatic relief of all these symptoms within as short a time as four hours. One of these patients, who had been unable to walk more than a few steps, walked two miles within twenty-four hours after the injection of 50 mg. of pyridoxine.

In view of Spies' results it seemed worth while to test the action of pyridoxine in patients suffering from clinical conditions characterized by extreme muscular weakness and exhaustion, in which no other signs or symptoms of a vitamin B complex deficiency were evident.

METHODS

Because of the difficulty of quantitating the patient's subjective feelings of muscular weakness, our tests were based on the ability of the individual to perform work to the point of exhaustion under standard conditions. For this purpose we employed two procedures:

(1) *Leg Test.*—With the patient in the supine position in bed, he was required to lift the extended right leg to the vertical position, at the rate of 24 times per minute, for as long as he was able to continue.

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(2) *Arm Test.*—In this test the work was done with the right hand and arm. While sitting up in bed, the patient was required to raise a 10-pound weight a distance of 13 inches, at a rate of 30 times per minute. He grasped a comfortable handle attached to a rope which ran over a pulley fixed at the foot of the bed. The resting position for this exercise was with the upper arm approximated to the lateral thoracic wall, so that the essential movement was a flexion of the forearm on the upper arm from the initial 90-degree angle to about a 30-degree angle.

The arm and leg tests were performed twice a day, with an intervening rest period of fifteen minutes. They were always conducted by the same observer in the same environment and at the same time of day. The tests were not considered satisfactory unless the results of two tests on the same limb coincided fairly closely. The average of these two tests was considered to be the work performance of the limb for the day. Preliminary experiments in normal people showed that the identical muscle testing procedures, performed daily, gave no indication of any increase in muscular ability due to training.

In order to avoid the possible psychologic effects of the therapy, all patients were given intravenous injections of saline daily from the beginning. When a constant base line of muscular ability had been established for a particular patient, a solution of pyridoxine hydrochloride* was substituted for the saline without the patient's knowledge. In some cases, after the results of the vitamin had been observed, the saline was again resumed as a further check upon the enthusiasm aroused in the patient by the beneficial effect of the treatment.

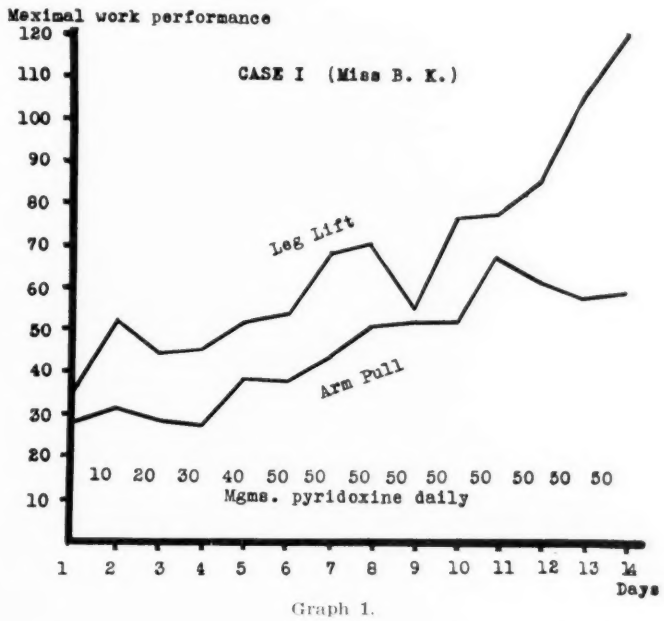
In our earliest cases varying doses of pyridoxine hydrochloride from 10 mg. to 50 mg. intravenously per day were used. We subsequently adopted a standard dose of 50 mg. a day for all cases for the purposes of this work. No toxic or ill effects of any kind were ever observed to occur from the use of these amounts of the vitamin.

The following is a summary of our results. Brief case histories are appended at the conclusion of this article.

Neurasthenia.—Our first case (Miss B. K.) was a 27-year-old female, whose ailment had never been definitely diagnosed. Physical and laboratory examinations had been consistently negative in the hands of numerous physicians, and at no time were any clinical signs of vitamin deficiency noted. She complained primarily of weakness and fatigue to such an extent that she had been practically bedridden for eight years. She was barely able to make the daily trip to the hospital for examination and treatment. We started this patient on pyridoxine hydrochloride without any preliminary tests with saline. Within five days she began to show increased muscular ability, as measured by our objective tests (Graph 1). Her appetite improved markedly and her weight rose from 99 to 104 pounds. After two weeks of treatment the patient stated that she was able to be up and out of bed for four hours a day. At this point we began to inject saline intravenously in place of the pyridoxine hydrochloride. Our objective tests showed no decrease in the patient's muscular strength, but she insisted that her weakness was returning and that she was no longer able to be

*Supplied by Merck & Co., through the courtesy of Dr. D. F. Robertson.

out of bed long enough to report for daily intravenous injection. We gave her 50 mg. tablets of pyridoxine hydrochloride to be taken orally, once a day at home, and advised her to return after two weeks. When next seen the patient reported that her strength was again returning. We now resumed the daily intravenous injections of pyridoxine hydrochloride. After fourteen more injections the patient was able to be up and about eight hours a day. We discontinued treatment and advised her to take fresh yeast at home. She has continued to improve and when last seen, five months after beginning pyridoxine, she was able to be out of bed eight hours daily, was attending a few social functions, and her weight had increased by 25 pounds.

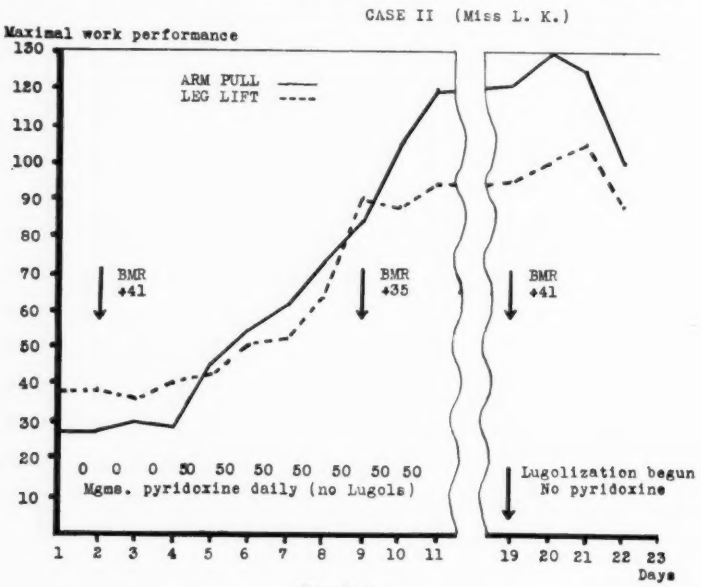


Graph 1.

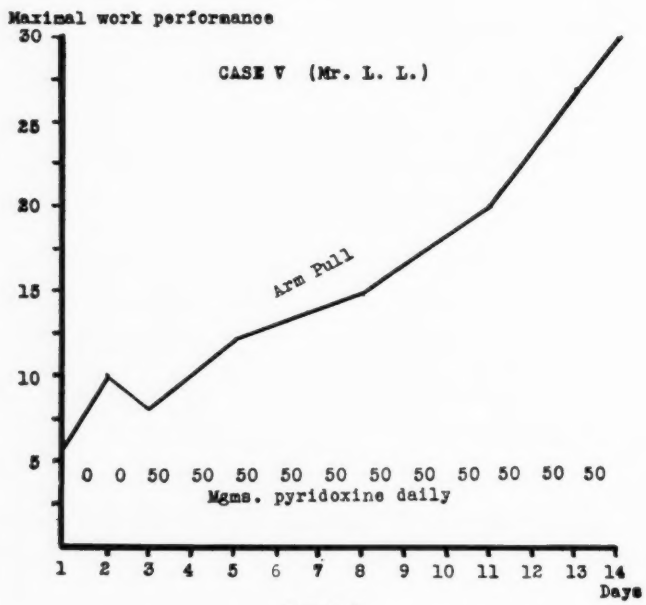
Hyperthyroidism.—We attempted the use of pyridoxine hydrochloride in three patients with hyperthyroidism, each of whom complained of marked weakness and fatigability. In every case there was a definite increase in muscular ability (Graph 2 illustrates one such case). Lugol's solution was withheld until we were able to demonstrate the effect of the vitamin. During this period the basal metabolic rates which were determined showed little change, so that the increase in strength could not be attributed to changes in the basal metabolic rate. In one case the pyridoxine hydrochloride was discontinued as soon as the patient showed increased strength and lugolization was begun. There was no further gain in strength. The Lugol's solution was continued and whole yeast was added to the diet. He immediately began to show further improvement as concerns muscular ability, although during this entire period his basal metabolic rate showed little variation.

Ulcerative Colitis.—Decided improvement in muscular ability was obtained with pyridoxine hydrochloride in 4 patients with ulcerative colitis. Objective tests could be made in only 2 of these patients, the others being unable to under-

take any exertion because of the presence of arthritis in one and severe cardiac damage in the other. Graph 3 summarizes the results in one of the cases (Mr. L. L.). The patient with arthritis (Mr. W. N.) who had been too weak to sit up in bed, proceeded to do so of his own volition and without help within sixteen



Graph 2.



Graph 3.

hours after receiving 50 mg. of pyridoxine hydrochloride intravenously. He even asked to be allowed to get out of bed. The patient with cardiac disease (Mrs. J. L.) had been at complete rest in bed for eight weeks, could not sit up without help and support, and had to be assisted in eating. Following three

days of pyridoxine hydrochloride administration, she was able to sit up in bed without support and to feed herself. After fourteen days of treatment she was able to begin walking. In no case were we able to note any improvement in the ulcerative colitis per se as regards frequency and number of stools.

Myasthenia Gravis.—Pyridoxine hydrochloride was of no apparent benefit in one case of myasthenia gravis which had been previously well controlled with prostigmin. For the purposes of observation, prostigmin was discontinued the previous night, and the patient was given 50 mg. of pyridoxine hydrochloride intravenously in the morning. By 6:00 P.M. the patient began to show acute signs of muscular weakness, as evidenced by her inability to eat or even swallow water. She was now given 100 mg. of pyridoxine hydrochloride intravenously, but showed no response. One-half hour later prostigmin was given, to which she responded almost immediately.

Malnutrition.—In 2 patients with anorexia nervosa and in one patient with cardiospasm we obtained no evidence of increased strength following the use of pyridoxine hydrochloride. All 3 patients were markedly underweight, were exceedingly weak, subsisted on deficient diets, and showed clinical signs of vitamin deficiencies. None of these patients showed any real response after receiving eight to fourteen daily injections of pyridoxine hydrochloride.

SUMMARY AND DISCUSSION

Noteworthy relief of muscular weakness has been obtained following the intravenous administration of pyridoxine hydrochloride in one patient with so-called neurasthenia, 3 patients with hyperthyroidism, and 4 patients with ulcerative colitis. No results were obtained in one patient with myasthenia gravis and in 3 patients with severe malnutrition. It is evident that our good results were obtained in patients in whom one might expect to find either a deficiency of vitamin intake or a vitamin requirement in excess of the normal intake, even though no recognizable symptoms of vitamin deficiency other than weakness were present. However, we cannot explain why the 3 patients with severe malnutrition with obvious manifestations of multiple vitamin deficiencies did not respond to pyridoxine hydrochloride. It is suggested that these failures might have been due to the absence of other vitamin factors which are essential to the action of pyridoxine hydrochloride.

CASE REPORTS

CASE 1 (Miss B. K.).—The essential details of the history and the results of treatment (Graph 1) with pyridoxine hydrochloride have been summarized above. It is worthy of note that this patient had been on a subminimal diet for eight years, eating from 800 to 1,000 calories a day, with very little meat and whole grain foods.

Physical examination showed a tall, thin female, 5 feet 9 inches in height, and weighing only 101 pounds. There were no other positive physical signs. Bronchoscopy showed an atrophic and hyperemic mucosa.

Laboratory Studies: Hemoglobin 80 per cent; erythrocytes 4,400,000; leucocytes 7,750 with a normal differential; urine negative; sugar 64 mg. per cent; nonprotein nitrogen 27 mg. per cent; chlorides 494 mg. per cent; cholesterol 298 mg. per cent; icterus index 6; carbon dioxide combining power 57.3 per cent; van den Bergh, direct and indirect, both negative; sputum negative for tubercle bacilli.

CASE 2 (Mrs. L. K.).—A white, divorced, 24-year-old female, entered the hospital with nervousness, weight loss, weakness, and prominence of the eyes for the past year.

Physical examination showed a marked exophthalmos, a diffusely enlarged thyroid gland, moist warm skin, a fine tremor of the hands, rapid pulse (90 to 110), and a blood pressure of systolic 130 and diastolic 70.

Laboratory studies were essentially negative, except for a basal metabolic rate of +41.

The report of the pathologist on the appearance of the thyroid after removal was consistent with the diagnosis of diffuse toxic goiter.

This patient received no Lugol's solution during the period of administration of pyridoxine hydrochloride, and her basal metabolic rate showed no noteworthy change. Nevertheless her muscular ability increased markedly, as shown in Graph 2.

At this time the patient refused to stay in the hospital because of difficulties at home. She received no medication while at home, and returned to the hospital one week later with the same signs and symptoms. Her basal metabolic rate was still +41. Lugol's solution (ten drops t.i.d.) was begun, without any pyridoxine hydrochloride. The patient showed no further increase in muscular ability, although her basal metabolic rate dropped to +13.

CASE 3 (Mr. S. D.).—A white, married, 35-year-old male, entered the hospital with nervousness, feeling of warmth and perspiration, palpitation, polyuria, diarrhea, enlargement of the neck, and the loss of 30 pounds of weight within two months.

Physical examination showed a slight exophthalmos, a diffusely enlarged thyroid, warm, moist skin, a fine rapid tremor of the hands, a pulse rate of 100, and a blood pressure of systolic 144 and diastolic 72.

The only significant laboratory test was a basal metabolic rate of +80.

The postoperative examination of the gland showed a diffuse toxic goiter, consistent with the clinical diagnosis of diffuse toxic goiter with hyperthyroidism.

This patient showed an increase in muscular ability immediately after his first dose of pyridoxine hydrochloride. After he had received five daily doses it was necessary to stop the pyridoxine and to start Lugol's solution because of his continued toxic state. However, by this time his arm pull had increased from 35 to 44, and his leg lift from 45 to 85. The basal metabolic rate taken on admission was +81, and after pyridoxine hydrochloride it was +72.

Five days after the beginning of treatment with Lugol's solution (10 drops t.i.d.) the basal metabolic rate dropped to +53, but there was no change in muscular ability. Whole yeast was then added to the diet, and within seven days the arm pull increased to 58 and the leg lift to 260. The basal metabolic rate at this time was +50.

CASE 4 (Mrs. B. R.).—A white, married, 38-year-old female, entered the hospital with nervousness, palpitation, a swelling in the neck, and weight loss over a period of three months.

There was a marked exophthalmos, a diffusely enlarged thyroid, a fine rapid tremor of the hands, warm moist skin, rapid pulse rate (105), and a blood pressure of systolic 140 and diastolic 80.

Repeated basal metabolic rates ranged from +80 to +40. Other laboratory studies were negative.

The diagnosis of diffuse toxic goiter with hyperthyroidism was confirmed by examination of the thyroid postoperatively.

This patient was given thiamin chloride, 15 mg. intravenously, for five days, but showed no increase in strength. Pyridoxine hydrochloride was then substituted for the thiamin chloride, and within ten days her leg lift increased from 40 to 250, and her arm pull from 16 to 85. Her basal metabolic rate remained unchanged during this period.

CASE 5 (Mr. L. L.).—A white, 19-year-old schoolboy, with a history of chronic nonspecific ulcerative colitis of three years' duration, entered the hospital complaining of an acute exacerbation of the colitis.

Physical examination disclosed an emaciated youth, with a right lower abdominal scar, the site of an appendectomy three years previously. Proctoscopic examination showed a hyperemic mucous membrane. Radiographic studies were consistent with a diagnosis of chronic ulcerative colitis.

No causative specific organisms could be found on repeated stool examinations. Hemoglobin was 50 per cent and erythrocytes were 2,890,000.

Medical treatment was attempted, consisting, over a period of several weeks, of a course of neoprontosil, repeated transfusions, iron, high vitamin high-caloric diet, and autogenous vaccine. The patient failed to show any improvement. An ileostomy was performed six weeks after admission.

We first saw the patient three months after admission. He was weak and emaciated; he had developed contractures of both legs and was having frequent, severe rectal hemorrhages.

Because of the state of his leg muscles it was possible to test only his arms. These showed the definite improvement in muscular ability summarized in Graph 3. This increase was paralleled by a general feeling of increased strength. At this time a hemicolectomy was performed and the patient died soon afterwards. Post-mortem examination showed a nonspecific colitis complicated by a diffuse peritonitis.

CASE 6 (Mr. W. N.).—A white, 26-year-old male, German refugee, had an eleven-year history of chronic ulcerative colitis. Seven years prior to his admission he had had an ileostomy performed in Germany with but little relief; he continued to have acute exacerbations of his colitis. When we first saw him, he had entered the hospital for the second time within a month. He was having five to six bloody mucous stools a day. He had developed a crop of furuncles over his legs and arms, and he had swollen, tender, reddened wrists and knee joints. He was extremely emaciated and so weak that he could not sit up in bed. Laboratory studies were negative. It was impossible to perform any objective tests because of the arthritis and marked weakness. He had been receiving thiamin chloride intravenously (15 mg. daily), to which we added pyridoxine hydrochloride (50 mg. daily). The next day he was sitting up in bed, asking to be allowed up, and his appetite was excellent. We continued to give the pyridoxine hydrochloride for one week. The patient continued to evidence increased strength, but neither his colitis per se nor his arthritis changed. Pyridoxine hydrochloride was discontinued after a week and fresh yeast (three cakes a day) was added to his diet. He was discharged two weeks later, his arthritic symptoms were gone, and his colitis improved. He was advised to continue taking yeast at home.

He re-entered the hospital six weeks later with a re-exacerbation of his colitis and arthritis. He had not taken any yeast because of the "gaseous feeling" which he claimed followed its use. On this admission the patient failed to show any further response to pyridoxine hydrochloride.

CASE 7 (Mrs. J. L.).—A 54-year-old white, married female entered the hospital with cramping abdominal pains and a diarrhea of two weeks' duration.

Physical examination was negative except for a rapid irregular pulse rate. Radiographic, proctoscopic, and laboratory studies established the diagnosis of nonspecific ulcerative colitis complicated by a chronic cystitis and an auricular fibrillation of undetermined etiology. The patient was treated symptomatically with sedatives, transfusions, and high caloric low-residue diet. On an empirical basis she was given a treatment with carbarsone and emetine hydrochloride, and later sulfanilamide therapy. To supplement her diet she was given thiamin chloride, 25 mg. intravenously, nicotinic acid 25 mg., b.i.d., cevitamic acid 200 mg., b.i.d., and liver extract 1 c.c. intramuscularly. In spite of these measures her condition remained unchanged.

When first seen by us, the patient had been in the hospital for eight weeks. Because of her cardiac condition and marked weakness, objective tests were impossible. Her weakness had progressed so far that she even required help to eat. We gave her intravenous saline for five days with no effects. We then started pyridoxine hydrochloride. The next day the patient felt much stronger, responded readily to questions, and noted an improvement in her appetite. By the time 150 mg. had been given (three daily doses) she was able to sit up in bed for five minutes without assistance. After ten doses she was sitting up in a wheel chair, and after two weeks of treatment she began to take a few steps; she was then discharged from the hospital. At no time during this period was there any change in frequency and number of stools.

CASE 8 (Mr. E. S.).—A white, married, 40-year-old male, when first seen had weakness and fatigue. Three months ago he had had a colostomy performed because of chronic ulcerative colitis. His colitis had improved, but he still complained of fatigue on slight exertion.

Physical examination was negative, except for the colostomy opening. Laboratory studies offered no aid.

This patient was started on pyridoxine hydrochloride and showed a gradual improvement in strength. At the end of ten days his arm pull had increased from 35 to 70 and his leg lift from 30 to 90. During two weeks of treatment he gained 10 pounds; in the previous three weeks he had gained only 3 pounds.

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COMPARATIVE EFFECTS OF PROPYLENE GLYCOL AND SOME OTHER AGENTS ON OXYGEN CONSUMPTION OF THE ORGANISM*

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THE voluntary running activity of white rats receiving propylene glycol was found previously to be increased somewhat.[1] These rats were not depressed, and they stored more glycogen than normal animals. The oxygen consumption of isolated liver perfused with propylene glycol was decreased, and the organ stored glycogen simultaneously.[2] These results are related to the possible food value of propylene glycol, which has a definite glycogenic action. But some increase in general activity should theoretically increase oxygen consumption or leave it unchanged. Therefore, it was thought desirable to investigate further the metabolic actions of this glycol, using oxygen consumption as the criterion. This report presents the results obtained.

METHOD

The apparatus used was of the closed-circuit type, a unit consisting of a Hempel desiccator acting as a rat chamber, which was connected by glass tubing and a three-way stopcock to a large glass cylinder of 4,500 c.c. capacity and

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graduated at 10 c.c. intervals. Soda lime and calcium chloride were put in the desiccator to absorb carbon dioxide and water. The large cylinder was filled with pure oxygen. As a rat used oxygen, water was allowed to siphon into the large cylinder from an 18-liter reservoir, thus equalizing the pressure inside the system with that of the atmosphere. The amount of water siphoned over was a measure of the oxygen consumed, after appropriate corrections for changes in temperature and barometric pressure. Six such units were operated at once.

The rats were observed for seven hours in this metabolic arrangement. No rat was used for more than four consecutive days. Usually 2 runs with the use of glycol or other agents were made, alternated with 2 control runs. The oxygen consumption was expressed as cubic centimeters of oxygen for 100 sq. cm. of body surface per minute. Since the metabolism varied with the environmental temperature, the control observations on each rat were plotted against the environmental temperature and the line through these points calculated by the method of least squares. The point at which this line crossed the 22°-axis was chosen to represent the average control result. Direct comparison of the results on controls and with different agents was made by moving the experimental points parallel to the control line until the 22°-axis was reached. The values so obtained were averaged, and the averages are summarized in Table I.

The nutritional state of the animals and the environmental temperature were as close to those of previous feeding and activity experiments as possible.[1] The animals were fed the usual stock diet of this department and were not fasted prior to the determination of oxygen consumption. The environmental temperature was not held constant but was carefully observed, and the mean values of oxygen consumption were corrected for the variations in temperature.

The doses of propylene glycol and other agents used and methods of administration were as follows: propylene glycol, 5 c.c. and 10 c.c. per kilogram, gastrically; benzedrine, 2 mg. per kilogram, subcutaneously; pentobarbital, 30 mg. per kilogram, subcutaneously; and chloral hydrate, 250 mg. per kilogram, intraperitoneally. The other agents, besides glycol, were used as a further check on the results with the glycol. Benzedrine, which is a general excitant, was expected to increase the oxygen consumption, and pentobarbital and chloral, depressants, were expected to decrease it. However, only the benzedrine acted according to expectations. A total of 505 determinations on 19 rats was made. The duration of each observation was seven hours. The other agents were compared in the same rats receiving the glycol.

CONTROL RESULTS

A total of 256 control observations on 19 rats showed an average oxygen consumption of 1.363 c.c. per 100 sq. cm. per minute at 22° C. (Table I). This value had a variability of less than 3 per cent.

GLYCOL AND OTHER AGENTS

Propylene glycol, in both doses used, depressed the oxygen consumption significantly, i.e., down to an average of 1.142 c.c. from the control average of 1.363 c.c. Benzedrine raised the oxygen consumption in 14 rats and increased

TABLE I

OXYGEN CONSUMPTION OF RATS RECEIVING PROPYLENE GLYCOL AND OTHER AGENTS

RAT	CONTROL			PROPYLENE GLYCOL				BENZEDRINE		PENTOBARBITAL		CHLORAL	
				10 C.C. PER KG.		5 C.C. PER KG.							
	DETERMINATIONS	O_2*	S.E.†	DETERMINATIONS	O_2*	DETERMINATIONS	O_2*	DETERMINATIONS	O_2*	DETERMINATIONS	O_2*	DETERMINATIONS	O_2*
1	8	1.502	±0.009	4	1.138			4	1.974				
2	9	1.285	±0.016	4	1.147			4	1.593				
3	7	1.431	±0.032	3	1.303			3	1.735				
4	9	1.327	±0.029	5	1.158			3	1.668				
5	22	1.315	±0.026	5	1.269	5	1.132	4	1.585	5	1.273		
6	6	1.324	±0.047	3	1.229			3	1.661				
7	18	1.249	±0.030	5	1.241	5	1.470	5	1.476	5	1.485		
9	19	1.275	±0.034	5	1.112	4	1.209	5	1.597	4	1.163		
10	19	1.252	±0.035	5	1.017	5	1.183	5	1.448	5	1.263		
11	15	1.300	±0.036	5	1.103	2	1.071	5	1.525	2	1.220		
12	19	1.457	±0.039	5	1.065	5	1.309	5	1.670	5	1.343		
14	20	1.348	±0.029	5	1.205	5	1.329	5	1.629	5	1.243		
15	21	1.426	±0.041			5	1.437			5	1.152	5	1.363
16	6	1.290	±0.086	4	0.917			2	1.377				
17	7	1.435	±0.038	4	1.099			3	1.560				
18	19	1.452	±0.036			5	1.355			5	1.475	5	1.400
20	11	1.242	±0.029			5	1.125			5	1.074		
21	11	1.352	±0.045			5	1.325			4	1.629		
22	12	1.650	±0.068			5	1.531			5	1.519		
	258	1.363		62	1.142	56	1.289	56	1.607	55	1.319	10	1.381

*Oxygen consumption = average c.c. oxygen consumed per 100 sq. cm. of body surface per minute at 22° C.
†Standard error of mean.

the activity, i.e., increased general excitability but did not cause tremors; the oxygen consumption was raised to 1.607 c.c. Interestingly enough, pentobarbital decreased the oxygen consumption only slightly in 12 rats. This was interesting, since the animals which received propylene glycol behaved in the same way as did the unmedicated controls and were not narcotized, whereas those receiving pentobarbital were markedly depressed and, in fact, comatose, although the reflexes could be elicited. Recovery occurred at the end of a seven-hour period. Therefore, the depression of oxygen consumption caused by propylene glycol was not due to a decrease in general activity. This confirmed the observations on the isolated perfused liver and explained why the rats, although showing no depression of running activity, were able to store energy (liver glycogen) better than control rats. The burning of propylene glycol requires less oxygen to release a given amount of energy than do most other foodstuffs (except fat).

The results in 2 rats with chloral, which markedly depressed these animals and caused coma with loss of reflexes, were also interesting in that the oxygen consumption was practically the same as that of the controls, contrary to expectations. However, the results with the agents, other than glycol, are of value in showing that the depressant effects of propylene glycol on oxygen consumption are peculiar to this agent, since all other agents tried, except benzedrine, compared favorably with the controls. The explanation of this effect of propylene glycol is not at hand, although the results in the whole animal are consistent with a similar depression of oxygen consumption of the isolated liver perfused with this glycol. This storage of glycogen in the isolated liver, as well as in whole animals, indicates storage of energy for which there is less need of oxygen, despite a fair general activity of the animals.

CONCLUSIONS

1. Propylene glycol was found to depress the oxygen consumption of white rats, this depression not being due to a decrease in activity, since the depressions of pentobarbital and chloral in the same rats did not significantly decrease the oxygen consumption.

2. These results on whole animals are in agreement with previous results on isolated liver perfused with propylene glycol, which also showed a decrease in oxygen consumption.

3. The doses of propylene glycol used were beyond those likely to be taken as solvent or vehicle in food or medicinal agents, but the depression of oxygen consumption could occur with very large or toxic doses. This result is essentially of scientific importance, because the use of propylene glycol has a wide margin of safety.

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CLINICAL CHEMISTRY

INSULIN FERRIHEMOCHROMOGEN*

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DURING the course of an investigation on the conjugation of ferriheme (hematin) with various nitrogenous substances in liquid ammonia (a process by which it is possible to prepare ferrihemochromogens which are soluble in aqueous media at physiologic pH) it was desired to study the reactions of ferriheme with a protein in this solvent. Insulin was used in this particular instance because it is one of the few proteins which is soluble in anhydrous ammonia.[1, 2] When it was discovered that certain insulin ferrihemochromogens formed by this reaction showed a modification of the characteristic physiologic activity of the hormone, this modification was studied. The results indicate for these soluble insulin conjugates some of the properties possessed by the protamine conjugate and other relatively insoluble depot forms of insulin.

Certain nitrogenous substances combine with ferriheme to give rise to a class of compounds called ferrihemochromogens. Among these nitrogenous substances termed "suitable" by Bertin-Sans and de Montessier, are ethylamine, ammonia, aniline, glycine and taurine,[3] nitrite[4] (in the nitro form), cetyl pyridinium chloride and nicotinamide[5] and cyanide (in the isonitrile form). Some nitrogenous substances are "unsuitable" in this sense; acetanilid and sulfanilamide[5] and triethanolamine[6] come into the latter category. The presence of quaternary nitrogen seems to be essential to the formation of a coordination compound with the iron of ferriheme.†

Basic proteins, which contain a large proportion of quaternary nitrogen, can combine with large amounts of ferriheme. Globin can, in fact, take up fully thirty times the quantity of heme as there exists, preformed, in hemoglobin. This capacity of globin is exceeded by that of insulin which was found to be capable of combination with a maximum of about eight times its weight of ferriheme chloride—a ratio of one iron atom to each nitrogen of the insulin molecule.

EXPERIMENTAL DATA

Crystalline insulin assaying 24 units per milligram or amorphous insulin assaying 20 units per milligram was dissolved in anhydrous ammonia in a

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†Another type of ferriheme combination, that with hydro-acid radicals of small ionic volume, is possible. These combinations are polar in nature and have been termed "hemoferrides."[7] The term "ferrihemide" would be more appropriate. "Suitable" ferrihemide formers may or may not contain nitrogen, and this need not be in the quaternary form, viz.; azide ion,[8] cyanide ion (in the nitrile form[9]) and cyanamide ion.[9] The combinations of insulin with ferriheme appear to be exclusively of the ferrihemochromogen type.

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pyrex Dewar flask and treated with various amounts of ferriheme chloride.[10] The ammonia was allowed to boil off at room temperature through a mercury trap, the last traces being removed by a suction pump. The insulin ferriheme conjugate was less soluble in anhydrous ammonia than either of its components, for, before all the ammonia boiled off, it precipitated as a gelatinous strawberry-colored mass. This became a greenish-black residue on removal of all the ammonia. This residue was dissolved in water to a final concentration of 10 units per cubic centimeter calculated on the basis of original insulin content.

TABLE I

WEIGHT OF RABBIT (KG.)	MATERIAL			TIME INTERVAL		BLOOD SUGAR (MG. PER 100 C.C.)	REMARKS
	INSULIN UNITS	INSULIN FERRIHEMOCHROMOGEN					
		RATIO	UNITS	HR.	MIN.		
2.88	10			0		97	Intravenously
				1	41	52	
				24	36	102	1 hour postprandial
3.05		1:6	15	0		99	Intraperitoneally
				1	21	81	
				24	26	81	1 hour postprandial
3.58		1:6	20	0		113	Intravenously
				1	29	56	
				24	40	77	1 hour postprandial
1.22		1:8	5	0		108	Intraperitoneally
				1	30	53	
				3	40	<45	Convulsions (20 c.c. 10% dextrose)
	5			22	40	75	Intraperitoneally
				23	10	<45	Convulsions
1.76		1:8	10	0		106	Intraperitoneally
				1	30	59	
				3	20	<45	Convulsions (20 c.c. 10% dextrose)
				22	40	56	Post-mortem sample Found dead in rigor

A few of the theoretically numberless pigment conjugates with varying insulin and ferriheme ratios were prepared. One with a high ratio (5:1 of insulin to ferriheme), one with a low ratio (1:6), and one which was completely saturated with ferriheme (1:8 insulin ferrihemochromogen) were tested for physiologic activity. The first showed no difference in activity from that exhibited by ordinary insulin when given in the same manner and dosage. Subcutaneous injection postprandially of 10 units in a 2.72 kg. rabbit caused convulsions in 208 minutes, while the control solution of ordinary insulin under the same conditions brought the blood sugar level to the convulsive level in a litter mate in 197 minutes. The effect of insulin-ferrihemochromogen ratios of 1:6 and 1:8 on the blood sugars of rabbits is given in Table I. There appears to be a definite prolongation of the insulin effect even when these conjugates are given intravenously or intraperitoneally, a condition which does not obtain when the depot forms of insulin are given in this manner.[11] The 1:8 ratio of insulin and ferrihemochromogen (which is fully saturated with the pigment

as evidenced by the settling from solution of small amounts of ferriheme) was also tested by the mouse method. Ten animals were convulsed in an average of seventy minutes while one-fourth the dosage of ordinary insulin caused convulsions in 10 mice in an average time of twenty minutes.

No toxic manifestations other than for the insulin effect have been noted in any of the animals used, some of which have been pushed to the convulsive level repeatedly by the administration of the insulin ferriheme conjugate over the past year.

The nitric oxide and carbon monoxide derivatives of 1:8 insulin ferrohemochromogen have been prepared. Their study is in progress and is of interest since they appear to be somewhat resistant to tryptic digestion. Attempts to form the cyanamide and the cyanide derivatives of 1:8 insulin ferrihemochromogen have not been successful.

SUMMARY

A series of water-soluble conjugates formed by the reaction of insulin and ferriheme chloride in anhydrous ammonia have been prepared. Insulin combines with ferriheme to a maximum ratio of 1:8 where a relationship of one insulin nitrogen to one ferriheme iron atom exists.

Chemical studies indicate that the combination is similar to that formed by nicotinates and ferriheme where the nitrogen of the former is coordinated in the 4_0 electronic orbit of the iron (ferrihemochromogen linkage) and is different from that formed between cyanamide and ferriheme where the nitrogen is polar covalent with the 3_2 orbit (ferrihemide linkage).

The molecular weight of 1:8 insulin ferrihemochromogen is calculated to be in the neighborhood of 315,000. Consequently, it resembles in physiologic action the "depot" forms of insulin, and, even with intravenous or intraperitoneal injection, administration to rats and rabbits results in hypoglycemia which is delayed in its onset and prolonged in duration.

Repeated administrations in rabbits have indicated neither acute nor chronic toxic effects other than for the hypoglycemic convulsions induced, and this is considered as evidence against the reputed toxicity of ferriheme (hematin).

I wish to acknowledge my indebtedness to Dr. R. G. Roberts, of the Chicago Medical School, and to Dr. Walter S. Bridgins, of Armour and Company, for their material help in the investigation.

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CLINICAL USE OF THE CEPHALIN-CHOLESTEROL FLOCCULATION TEST*

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WE BECAME interested in the cephalin-cholesterol flocculation test, as developed by Hanger,[1] because of the simplicity of this method for evaluating "irritation of liver cells" and the significance of clinical jaundice. We have had the opportunity to study the test in 114 patients and present the results as indicating that the test can be of definite clinical value.

Recently Rosenberg[2] has reported a considerable series which confirms the statements of Hanger. In 155 selected cases, he found that the test was reliably correlated with the clinical findings and felt that negative or slight reactions are indicative of obstructive jaundice, while prompt and strong reactions are indicative of parenchymatous jaundice.

The test is performed by adding to 4 c.c. of normal saline solution 1 c.c. of cephalin-cholesterol suspension[3] and 0.2 c.c. of the serum to be tested. After mixing, the tube is allowed to stand at room temperature for twenty-four hours, then read. A homogeneously cloudy solution is read as a negative test. Complete flocculation and precipitation, with a clear supernatant fluid, is read as four-plus. We have considered three-plus and four-plus tests as "positive" and one-plus and two-plus tests as "doubtful."

For confirmation, as many tests as possible were performed on each patient. In most cases, the icterus index, total protein and partition, and total cholesterol were determined, and in many cases bromsulfalein or galactose tolerance tests were done as well. It was impossible to obtain pathologic or operative proof in all cases, but cases were excluded from consideration unless the diagnosis was reasonably established.

In Group I, there were 83 cases with no clinical or laboratory evidence of intrinsic liver disease or of biliary obstruction. Of these, only 4 showed positive tests. Two were in cases of hyperthyroidism, one coincident with severe pernicious anemia. One was in a case of Cooley's anemia with a markedly enlarged liver. The fourth was a case of bronchial asthma and probable periarteritis nodosa. In each of these it is conceivable that liver damage or irritation was present in the absence of other positive tests. It is well known that

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The cephalin-cholesterol mixture used in our tests was kindly furnished by Dr. F. M. Hanger. Method of preparation can be found in the literature.[1, 2]

liver damage occurs in hyperthyroidism. There were 12 more cases with doubtful tests. Of these, 3 were cases of hyperthyroidism, and one was a case of primary disseminated arteritis. In some of these repeated tests were later negative.

In Group II there were 22 cases with evidence of intrinsic liver disease. The 4 cases of catarrhal jaundice were all positive and became negative with the subsidence of clinical jaundice. Of the 6 cases of portal cirrhosis, 5 were positive and one was doubtful. Of 10 cases of longstanding chronic passive congestion due to cardiac failure, 4 were positive and 2 doubtful.

In Group III there were 9 cases of obstructive jaundice. Two were strongly positive. In one of these the test was taken just before exitus, after many months of increasing jaundice. The other was in a case where the jaundice was of only three weeks' duration; at operation a carcinoma of the pancreatic head was found. These results are compatible with those of Pohle and Stewart,[3] who found that the flocculation test was positive in 18 of 23 cases of obstructive jaundice. This may be explained on the basis of damage to the liver, which occurs with long-standing biliary obstruction or with ascending infection and hepatic damage accompanying obstruction.

TABLE I

	0 NEGATIVE	1-2 PLUS DOUBTFUL	3-4 PLUS POSITIVE
I. No liver disease or biliary obstruction	67	12	4
Cholecystitis	10	2	0
Hyperthyroidism	3	3	2
Others	54	7	2
II. Intrinsic liver disease	4	4	14
Catarrhal jaundice	0	0	4
C.P.C. of liver	4	2	4
Portal cirrhosis	0	1	5
Others	0	1	1
III. Obstructive jaundice	7	0	2
Carcinoma of head of pancreas	5	0	2
Common duct stone	2	0	0

CONCLUSIONS

We feel that the cephalin-cholesterol flocculation test is of definite use in the evaluation of clinical jaundice and hepatic disease. It is no more infallible than any other test of liver status. However, in doubtful cases and in "false" positives, there is usually a factor which makes the existence of subclinical hepatic damage or irritation (by other testing methods) a possibility.

That the test is exceedingly inexpensive and simple to perform makes its value greater, and we suggest that this test be used routinely in the study of cases with jaundice or with suspected hepatic disease.

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RETENTION OF NONPROTEIN NITROGEN IN THE BLOOD OF THE RABBIT FOLLOWING INTRAVENOUS INJECTIONS OF A HYPERTONIC SOLUTION OF DEXTROSE*

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RENAL lesions have been observed in both man and experimental animals following the intravenous injection of hypertonic solutions of sucrose.[1-3] Recently Rigdon and Cardwell[4] studied these lesions and found that the primary changes in the kidney occurred in the epithelial cells in the convoluted portion of the tubules. These cells were swollen, and their cytoplasm stained very lightly with eosin. The nucleus was shrunken, and the periphery was serrated. There was a retention of nonprotein nitrogen in the blood of the rabbits showing these histologic changes.

Investigators have usually found it difficult to produce renal lesions by the intravenous injection of hypertonic sucrose with any degree of regularity.[1, 2] Rigdon and Cardwell[4] observed that many of their clinical cases were dehydrated when they were first given the hypertonic solution of sucrose. In view of this clinical observation, these investigators dehydrated rabbits before and during the period in which the sucrose was given. With this technique they were able to produce renal lesions routinely. Accompanying this lesion there was a retention of nonprotein nitrogen in the blood.

It was suggested by Rigdon and Cardwell[4] that this renal lesion resulted from a retention of sucrose and water in the cytoplasm of the epithelial cells in the convoluted portion of the renal tubules. The nuclei of these cells were shrunken as the result of dehydration. The swollen epithelial cells sometimes completely blocked the lumina of the tubules. This process by which retention of nonprotein nitrogen occurred following the intravenous injection of a hypertonic solution of sucrose was reversible as shown by a diminution in the quantity of nonprotein nitrogen that was present in the blood of these rabbits following the addition of water and cabbage to their diet.

In the present experiments a hypertonic solution of dextrose was given intravenously to rabbits to study its effect on the kidney and on the retention of nonprotein nitrogen in the blood. The technique was the same as that used in previous studies when a hypertonic solution of sucrose was given to rabbits.[4]

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The nonprotein nitrogen determination was made in the chemistry laboratory of the department of medicine. I wish to thank Dr. Diggs for the use of his laboratory facilities.

TABLE I

RETENTION OF NONPROTEIN NITROGEN IN THE BLOOD OF RABBITS GIVEN A 50.0 PER CENT SOLUTION OF DEXTROSE INTRAVENOUSLY

	QUANTITY OF NONPROTEIN NITROGEN IN THE BLOOD (MG. PER 100 C.C.)																				
	OATS														OATS, CABBAGE, WATER					OATS		
EXP. DAY	800	801	802	803	808	809	900	901	928	929	930	931	978	979	902	903	933	934	935	806	807	980
1									33	37							35	33				
3	121*	53	44*	67	86	75	60	67	121	69.5	80.5*	69.5	55	60	50	67	52	54	42	50	67	50
4		88			78	78	92	75	148†	107		87.5†		113*	60	67	67.5	58.5	52	60	57	
4		120*								229*			*				47*					
5												72			75	67		58	58.5	60	60	
6					78*	120	115	150	92						63	67		48	44	55†	60	
7						171*	120†	300*	71.5			67.5				60‡					63*	
7									42			37						34	33			
10							67		34			33			40			27	32*	43		
11															38					33		
17							40															

All rabbits given only oats for three days before experiment begun.

*Rabbits either died or were killed at this time.

†Rabbits given cabbage, water, and oats at this time to see if the N.P.N. would return to normal.

Rabbits 800, 801, 802, 803, 928, 929, 930, 931, 933, 934, 935, and 979 received 25 c.c. of dextrose each day for three successive days.

Rabbits 808, 809, 900, 901, and 978 received 25 c.c. of dextrose each day for five successive days.

Rabbits 806, 807, and 980 were controls; they received no dextrose.

METHODS AND MATERIALS

Normal adult rabbits were used. They were given only oats for three days. At this time one group was given water, cabbage, and oats. A second group was kept on a diet of only oats. All of the rabbits were given 25.0 c.c. of a 50.0 per cent buffered solution of dextrose* (*d*-glucose) intravenously each day for three to five days. The controls were given only oats and no dextrose during the time of the experiment.

The nonprotein nitrogen and urea nitrogen content of the blood were determined by standard methods at intervals during the experiment. The rabbits were killed at varying times, and the kidneys were placed immediately into a solution of 10.0 per cent formalin. Paraffin sections were made and were stained with hematoxylin and eosin.

The rabbits were weighed at frequent intervals. When some of the rabbits, given only oats and dextrose, showed a retention of nonprotein nitrogen, they were given cabbage and water. This procedure was used for those rabbits in which the function of the kidney was permitted to return to normal as indicated by the level of nonprotein nitrogen in the blood.

EXPERIMENTAL DATA

Fourteen rabbits were fed only oats and were given dextrose intravenously. Each of the animals that lived for either four days or longer showed a retention of nonprotein nitrogen in the blood. Rabbits 900, 928 and 931, as shown in Table I, were given cabbage and water after they developed a retention of nonprotein nitrogen. The nonprotein nitrogen in Rabbit 900 was 120 mg. per 100 c.c. on the seventh day and on the seventeenth day of the experiment it had returned to within the range of normal. The nonprotein nitrogen in Rabbit 928 was 148 mg. per 100 c.c. on the fourth day of the experiment, and the nonprotein nitrogen in Rabbit 931 was 87.5 mg. per 100 c.c. On the eleventh day of the experiment the former rabbit's nonprotein nitrogen was 34 mg. per 100 c.c., and the latter's was 33 mg. per 100 c.c.

Five rabbits as shown in Table I were given cabbage, oats and water during the period in which the hypertonic solution of dextrose was injected. There was only a slight rise in the level of the nonprotein nitrogen in any of these rabbits. Rabbits 902 and 903 showed the greater degree of retention. They did not receive adequate amounts of cabbage during the time of the experiment.

Three rabbits (Table I) were given only oats, and the nonprotein nitrogen was determined at frequent intervals. The highest level of the nonprotein nitrogen was 67 mg. per 100 c.c. on the third day in Rabbit 807 and 60 mg. per 100 c.c. in Rabbit 806 on the sixth day. The results of this experiment illustrate the effect of dehydration on the retention of nonprotein nitrogen in the blood of the rabbit.

Urea nitrogen determinations were made in Rabbits 978, 979, and 980. They were 25.0 mg. per 100 c.c. in Rabbit 978 on the second day of the experiment, 28.0 mg. per 100 c.c. in Rabbit 979, and 22.0 mg. per 100 c.c. in Rabbit

*The dextrose was supplied by Eli Lilly and Company.

980. The urea nitrogen was 50.0 mg. per 100 c.c. in Rabbit 978 on the third experimental day. It was 60.0 mg. per 100 c.c. in Rabbit 978 on the fourth and only 30.0 mg. per 100 c.c. in the control, Rabbit 980. Rabbit 980 had only 25.0 mg. per 100 c.c. urea nitrogen on the fiftieth day. These observations show that the urea nitrogen of the blood increases in the rabbit following the intravenous injection of a 50.0 per cent solution of dextrose when the animals are dehydrated.

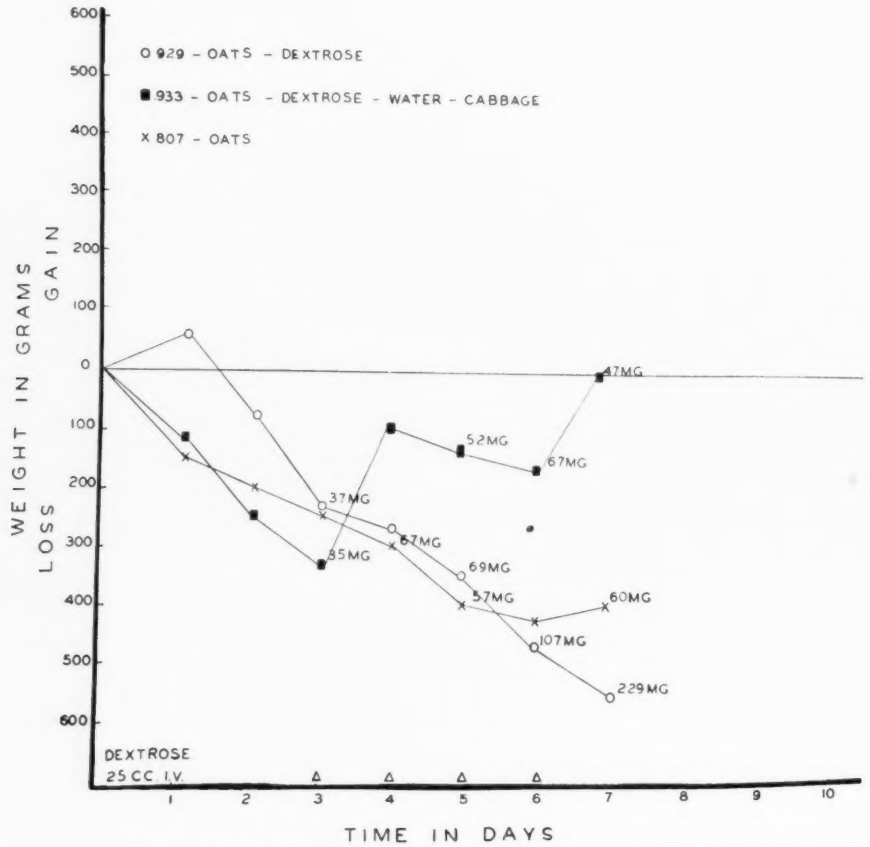

Fig. 1.—The graph illustrates the loss in weight and the amount of nonprotein nitrogen in the blood of the rabbits at different intervals during the experiment.

All of the rabbits lost weight during the time of the experiment. The average decrease in weight of the group given cabbage, water, oats, and the hypertonic solution of dextrose was 60 Gm. Those given only oats lost an average of 389 Gm. Fig. 1 illustrates the loss in weight of the rabbits and the amount of nonprotein nitrogen in the blood at varying intervals during the experiment.

Pathologic studies were made on the kidneys from Rabbits 801, 807, 903, 929, 933, 935, 978, and 979. Each of these animals was killed. The kidneys from Rabbits 809 and 901 were also studied histologically. These rabbits were dead for two hours or less before the kidneys were removed. There were no macroscopic lesions observed in the kidneys. The epithelial cells in the con-

voluted portion of the tubules in Rabbit 929 were slightly swollen and the cytoplasm was filled with small pink granules. A small amount of albumin was present in the lumina of the tubules. The kidney from Rabbit 807 showed essentially the same characteristics as the kidney from Rabbit 929. The epithelial cells in the kidney of Rabbit 979 were swollen, and the cytoplasm stained lightly with eosin. The kidneys from the other rabbits showed only some swelling of the epithelial cells in the convoluted portions of the tubule. In fact, there was very little histologic difference in any of these sections. The kidneys from the rabbits showing a retention of nonprotein nitrogen were very similar to the controls and also to those in which the level of nonprotein nitrogen was elevated and then returned to normal.

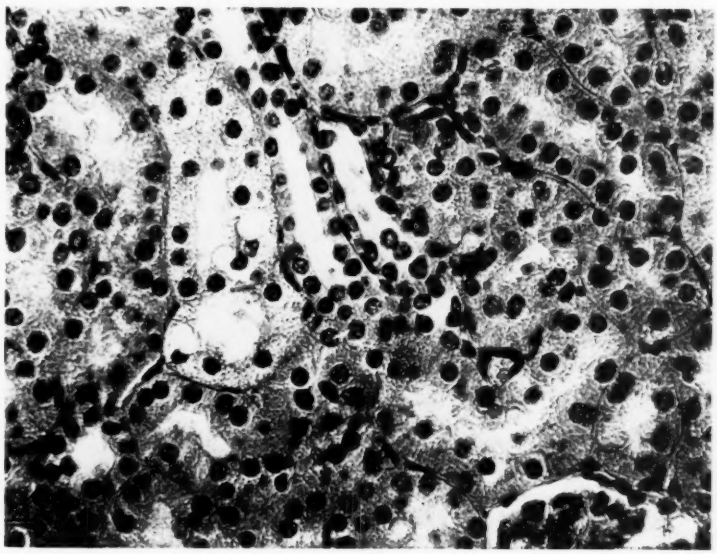

Fig. 2.—Rabbit 979. The epithelial cells in the convoluted portions of the tubules are swollen. Usually the cytoplasm is finely granular and stains deeply with eosin. This rabbit was given 25.0 c.c. of a 50.0 per cent solution of dextrose daily for three days. The nonprotein nitrogen on the fourth day at the time of death was 113 mg. per 100 c.c.

DISCUSSION

The data obtained from this study show that rabbits given 3 to 5 intravenous injections of a hypertonic solution of dextrose (25.0 c.c. of a 50.0 per cent solution) develop a retention of nonprotein nitrogen when the intake of fluid is restricted. Following the retention of the nonprotein nitrogen, if the animals are given fluids in the form of water and cabbage, the level of the nonprotein nitrogen in the blood rapidly returns to normal. This observation indicates that the mechanism by which the nonprotein nitrogen is retained in the blood is a reversible process. We are unable to give the maximum level which nonprotein nitrogen may reach and still return to normal.

No characteristic pathologic changes were observed in the kidneys of those rabbits that developed a retention of nonprotein nitrogen following the intravenous injection of a hypertonic solution of dextrose. The epithelial cells of the convoluted portion of the tubules may become slightly swollen and the cytoplasm may become granular. Small amounts of albumin were present in

a few of the tubules. The kidneys from the rabbits given oats and those from the rabbits given dextrose, oats, water, and cabbage showed essentially the same histologic changes.

The rabbits given hypertonic solutions of sucrose had swollen and vacuolated epithelial cells in the convoluted portion of the renal tubules. The nuclei of the epithelial cells were shrunken.[4] It was interesting to observe the difference in the histologic changes in the kidney in the rabbits given the hypertonic solution of sucrose and in those given the hypertonic solutions of dextrose. There was, however, a retention of the nonprotein nitrogen in the blood of the rabbit following the intravenous injection of both of these sugars.

Oats were selected for the basic diet of these rabbits since the amount of fluid obtained from them is only negligible. Cabbage was used primarily for its high-water content.

It would be interesting to know if there are any clinical complications following the use of hypertonic solutions of dextrose similar to those observed following the intravenous injection of hypertonic solutions of sucrose. Hypertonic solutions of dextrose are frequently used clinically for the reduction of intracranial pressure. Masserman[5] observed the effect of various amounts and different concentrations of dextrose on 85 normal patients. He found that "the administration of 50 Gm. or less in 20 per cent solution produced no untoward clinical sequelae other than diuresis; however, the intravenous injection of 100 Gm. or more in 30 to 50 per cent solution caused headaches and other adverse symptoms in 72 per cent of cases, whereas 58 per cent of the patients receiving 185 Gm. or more suffered transient pyrexia." Blau[6] has suggested that a hypertonic saline solution is preferable to hypertonic dextrose to reduce cerebrospinal pressure.

SUMMARY

Rabbits when dehydrated and given a daily injection of 25.0 c.c. of a hypertonic solution of dextrose for three to five days developed a retention of nonprotein nitrogen and urea nitrogen in the blood.

Following the retention of nonprotein nitrogen, when fluids were given, the nonprotein nitrogen content of the blood returned to normal.

There were no characteristic pathologic lesions observed in the kidney of the rabbits given solutions of hypertonic dextrose and developing a retention of nonprotein nitrogen.

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CONCENTRATION OF SULFAGUANIDINE (SULFANILYLGUANIDINE) IN THE PERIPHERAL BLOOD OF HUMAN BEINGS AFTER ITS INTRODUCTION INTO THE LARGE BOWEL AND AFTER ORAL ADMINISTRATION*

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SULFAGUANIDINE (sulfanilylguanidine), a sulfonamide derivative, has been described by Marshall and his associates[1] as a chemotherapeutic agent for intestinal infections. Relatively small amounts of the drug are absorbed from the gastrointestinal tract, thus providing a rather high concentration in the intestine where it may act on the coliform bacilli of the intestinal flora. It has been shown[1] that in dogs sulfaguanidine is absorbed to a much greater degree by the small bowel than by the large bowel and that the levels of the sulfaguanidine in the blood after absorption from the large bowel are much lower than the levels of sulfanilamide when the latter drug is given by the same route.

Five patients with well-functioning colostomies and with clinically and grossly normal gastrointestinal tracts above the site of obstruction afforded the opportunity of comparing the blood levels of sulfaguanidine which might result from its oral administration with those found after the drug was instilled into the large bowel in human beings. The procedure to be described was carried out after the colon had emptied spontaneously in the usual manner and at a time when no further activity of the large bowel was expected for at least six to eight hours. Doses of 3 to 5 Gm. (60 or 100 mg./kg. of body weight) of sulfaguanidine† were used in the form of suspensions and were introduced into the large bowel in a manner not liable to initiate peristalsis. The suspension was introduced into the distal loop of a transverse colostomy in 2 patients, into the proximal loop of a sigmoid colostomy in 2 patients, and into the distal loop of a sigmoid colostomy in one patient. Blood samples were collected at intervals during the following four to six hours. At the end of these periods of sampling, the dressing covering the colostomy was inspected to detect evidence that the suspension had been expelled. The loop of large bowel was then flushed with warm water to remove as much of the drug as possible. A dose of the drug similar to that placed in the large bowel was then given by mouth, and blood samples were collected at intervals during the subsequent seventeen hours. The levels of free sulfaguanidine in the blood samples were determined according to the method described by Marshall and his co-workers.[2]

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†Sulfaguanidine through the courtesy of Lederle Laboratories, New York.

The results are shown in Table I, and the trends of the concentration of the drug in the blood are represented in Fig. 1. There was no evidence that the drug had been expelled from the colon, yet a measurable concentration of sulfaguanidine was not found in the peripheral blood of any of the 5 patients during the four to six hours following its introduction into the large bowel. Concentrations

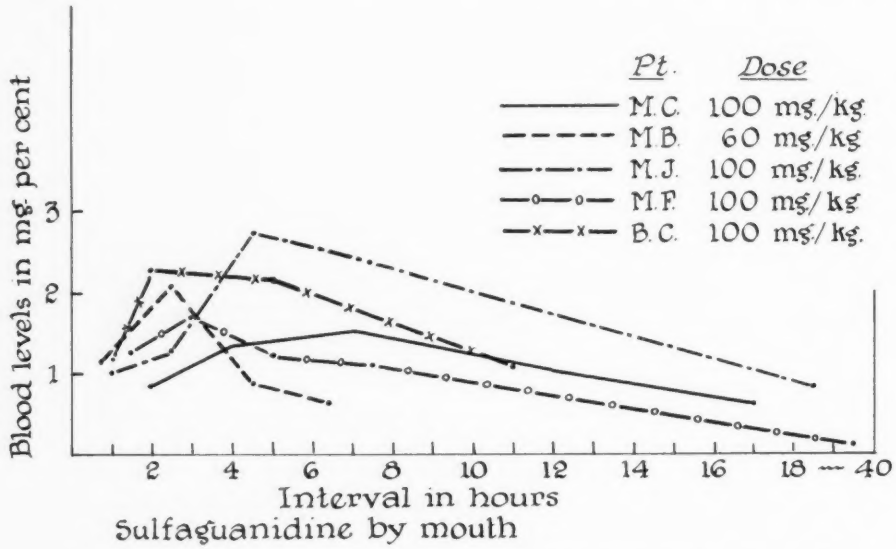

Fig. 1.—Sulfaguanidine levels in the peripheral blood after oral administration.

TABLE I

BLOOD LEVELS OF SULFAGUANIDINE AFTER ITS INTRODUCTION INTO THE LARGE BOWEL AND AFTER ORAL ADMINISTRATION

PATIENT	M. B.		M. C.		M. F.		M. J.		B. C.	
Large Bowel										
DOSE	60 MG./KG.		100 MG./KG.		100 MG./KG.		100 MG./KG.		100 MG./KG.	
	HR.	MG. %	HR.	MG. %	HR.	MG. %	HR.	MG. %	HR.	MG. %
	1	0.0	1½	0.0	1½	0.0	2	0.0	1	0.0
	3	0.0	3½	0.0	4½	0.0	5	0.0	3	0.0
	5	0.0	5½	0.0					4	0.0
*After Oral Administration**										
DOSE	60 MG./KG.		100 MG./KG.		100 MG./KG.		100 MG./KG.		100 MG./KG.	
	HR.	MG. %	HR.	MG. %	HR.	MG. %	HR.	MG. %	HR.	MG. %
	2	0.89	1	1.18	2	1.19	2	0.90	1	1.14
	4	1.42	2	2.13	4	1.60	4	1.37	3	2.28
	7	1.60	5	0.94	6	1.20	6	2.62	5	2.05
	12	1.06	7	0.70	8	1.02	9	2.40	11	1.14
	17	0.71	10	0.00	18	0.23	18	0.91		
			17	0.00						

*Sulfaguanidine removed from colon by lavage.

of the free form of the drug, varying from 0.89 to 2.13 mg. per cent, were found in the peripheral blood in all 5 of the samples withdrawn two hours after oral administration. The peaks of concentration occurred within two to seven hours, and the maximal blood level was 2.62 mg. per cent. In general, the blood levels corresponded to the dosage, being higher when larger amounts of the drug were given.

Following the oral administration of the drug, one patient complained of vertigo which lasted for twenty-four hours and another was nauseated for six hours. In 3 of the patients erythrocyte and leucocyte counts and hemoglobin determinations were made at the beginning and at the end of the experimental period. No significant differences were found.

CONCLUSION

Sulfaguanidine, in the form of an aqueous suspension, when introduced directly into the large bowel through a colostomy stoma, is not absorbed from the left half of the colon in human beings.

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THE IMMEDIATE EFFECT OF CIGARETTE SMOKING UPON BASAL METABOLIC RATES OF UNIVERSITY MEN AND WOMEN*

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THE object of this investigation was to determine the immediate effect of cigarette smoking upon the basal metabolic rate of the smoker, since a question as to the existence of such an effect had arisen. In spite of a careful search of the literature, little definite evidence was found. There seems to be, however, a general impression among clinical workers that smoking is followed by a rise in basal metabolism. We resolved to test this assumption under conditions approaching those under which clinical determinations are made. It was hoped that a definite answer to the question of the immediate effect of smoking could be obtained from the results.

Males[1] noted that the injection of rats with minimal doses of nicotine, the active principle of cigarette smoke, produced a transient rise in metabolic rate, followed by a more prolonged period of subnormal metabolic activity. This may be taken as an indication that smoking is capable of affecting the metabolism in some way. Furthermore, Schlumm[2] found a high basal metabolic rate (average +19.9 per cent by Harris-Benedict standards) in heavy smokers in Germany; the basal metabolic rates fell to a normal level soon after smoking was discontinued. More recently, Hiestand, Ramsey, and Hale[3] reported further evidence for an increase in basal metabolism as an immediate result of cigarette smoking. There is, therefore, some indication in the literature that smoking may affect the metabolic rate.

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EXPERIMENTAL

The subjects taken for these tests were men and women of college age, selected at random from students apparently in good physical condition at the time of the experiment. All were accustomed to smoking either habitually or occasionally. The subject relaxed for one-half hour before the first test; the control basal rate was then determined, using the Benedict-Roth recording metabolism apparatus. Two six-minute tests were averaged for the control run. Following this, the subject was allowed to sit up and smoke approximately three-fourths of a cigarette. Physical exertion was kept at a minimum. The subject then lay down again for another rest period of twenty-five to thirty minutes, at the end of which time two more six-minute runs were taken. From the average of these, the apparent basal metabolic rate after smoking was determined. The deviation from the control test, previously obtained, was expressed as percentage difference. The calculations of the normal calories per hour were made by use of the standards of Dubois, as modified by Sandiford and Boothby.

TABLE I

SUBJECT	SEX*	SMOKING HABITS†	BASAL RATE PER CENT	NORMAL CAL./HR.	ACTUAL CAL./HR. (CONTROL)	AFTER SMOKING CAL./HR.	PER CENT DEVIATION
V. Bu.	F	H	- 2.5	56.8	55.4	64.1	+15.7
M. C.	F	O	-10.0	54.2	48.8	55.9	+14.6
A. T.	F	O	- 9.8	59.0	53.2	59.4	+11.7
S. C.	F	O	-10.6	56.5	50.5	55.5	+ 9.9
F. J.	F	O	-14.1	59.0	50.7	53.4	+ 5.3
J. W.	M	O	-15.6	74.6	63.0	66.2	+ 5.1
J. V.	M	H	-13.5	70.9	61.3	64.0	+ 4.4
D. F.	F	O	- 8.9	60.9	55.5	57.3	+ 3.2
J. O.	M	H	-15.9	66.8	56.2	57.9	+ 3.0
S. L.	M	H	-14.6	78.1	66.7	67.9	+ 1.8
E. B.	F	O	- 8.8	53.5	48.8	49.7	+ 1.8
D. C.	F	H	-17.5	59.0	48.7	49.3	+ 1.2
J. C.	F	H	- 3.4	58.3	56.3	56.3	0.0
E. S.	F	H	- 6.6	58.8	54.9	54.0	- 1.6
J. F.	M	H	- 7.9	78.7	72.5	70.6	- 2.6
M. R.	F	O	- 4.7	55.0	52.4	50.7	- 3.2
M. S.	F	H	- 5.0	58.3	55.4	53.5	- 3.4
D. U.	M	H	- 3.6	75.4	72.7	69.4	- 4.5
V. Be.	F	H	- 0.8	59.0	58.5	54.1	- 7.5
J. G.	M	H	-14.3	72.9	62.5	57.3	- 8.7
Mean value							+ 2.3

*M = Male.
F = Female.
†O = Occasional smoker.
H = Habitual smoker.

Standard precautions and procedures (i.e., testing for leaks, determining absorption of carbon dioxide, etc.) were used throughout the experiment. The subject was, of course, in basal condition and had not smoked on the morning of the tests. In order to control a factor which might possibly vary, the same brand of cigarettes was used in all cases. It was shown at one time by the Bureau of Investigation of the American Medical Association[4] that this brand contained 1.44 per cent nicotine, which was slightly below the average for standard brands. The necessity for this precaution is indicated by the work of

Haag,[5] who found that the blood pressure of anesthesized dogs, given intravenous injections of smoke solutions prepared from several brands of cigarettes, could be correlated with the nicotine content of the cigarettes.

The 20 subjects were selected from more than 40 upon whom control tests were run. Only those tests in which, by all criteria, the subjects appeared relaxed, and in which the oxygen consumption in the two six-minute periods agreed within 3 mm. on the chart were considered. Results of doubtful validity were not used.

The results of the tests are shown in Table I. The last column was obtained by dividing the difference between the two previous columns by the control (actual calories per hour) and multiplying by 100.

DISCUSSION

The results given here shown that the apparent basal metabolic rate determined approximately one-half hour after smoking may be either above or below the control test taken prior to smoking, but that the deviation is by no means constant. In many cases the difference obtained was certainly great enough to be considered significant.

The work of many investigators has indicated that smoking, or nicotine, stimulates the adrenal glands to an increased secretion of adrenaline. Houssay and Molinelli[6, 7] showed that nicotine acts directly upon the adrenal medulla of dogs, causing release of the hormone by a specific reaction with that tissue. Haggard and Greenberg[8] found a rise in blood sugar after smoking, which they attributed to the action of adrenaline. Johnson and Short[9] observed a rise in the peripheral skin temperature after smoking, while Short and Johnson[10] noted that pulse rate, blood sugar, peripheral skin temperature, and blood pressure were similarly influenced by smoking and by small amounts of adrenaline.

In the recent report of Hiestand, Ramsey, and Hale[3] an adrenaline effect was pointed out as the probable cause of the physiologic responses to smoking. In 82 per cent of their subjects (not in basal condition) the metabolic rate was increased following smoking. Sixty per cent of our subjects showed an increased basal metabolic rate, whereas 35 per cent showed a lowering in the rate. The mean value of the percentage deviation was +2.3 in our study, while the above authors reported an average of +8.9. They did not attempt to explain the lowering of the metabolic rate, immediately following smoking, in 13 per cent of their subjects.

As Haggard and Greenberg[8] showed, when the blood sugar is raised, an increment in carbohydrate oxidation, and, therefore, a higher metabolic rate was to be expected. For this reason, a rise in basal metabolism following smoking was anticipated. However, a possible explanation for the unexpected variation in results has been tentatively advanced. It is well known that the sympathetic nervous system varies in responsiveness in different individuals, and that the adrenals are under its control. It is suggested that the response of these glands to the stimulation due to smoking occurs after different time intervals in different subjects, causing a preliminary rise in basal metabolic rate, followed by a drop

to subnormal values before the return to normal. This supposition is borne out by the work of Males[1] on rats. Johnson and Short[9] noted a time difference in their subjects in changes of the peripheral skin temperature after smoking, and Hiestand, Ramsey, and Hale[3] noted changes in responses of their subjects over a period of forty-five minutes following smoking, with regard to heart rate, blood pressure, oxygen pulse, and metabolic rate.

Haggard and Greenberg[8] observed that the respiratory quotient reached its peak in about fifteen minutes after smoking, and during the next thirty minutes returned to the normal level or slightly below. As the method of indirect calorimetry was used in our determinations, it is evident that in cases of apparent increase in the basal metabolic rate, the actual increases may have been even greater than those calculated, since the method of calculation assumes a respiratory quotient of exactly 0.82. The work of Dill, Edwards, and Forbes[11] on ten subjects, however, indicated that the smoking of one cigarette resulted in no appreciable change in respiratory quotient.

With regard to interpreting our own results, therefore, it is possible that (1) the "metabolic rate-time curve" may have been distinct and characteristic for each person; and (2) the basal metabolic rates of our subjects were determined at different points on their individual curves.

With regard to other phenomena observed, it was found that the pulse rate was generally higher, the body temperature usually higher or constant, and the rate of respiration often higher after smoking. This is in general agreement with the work of Short and Johnson and others, although Hiestand, Ramsey, and Hale found a decrease in the rate of respiration. It was further noted that sex seemed to have no influence upon the immediate reaction to smoking.

It was significant that all the control basal metabolic rates of our subjects showed a negative deviation from the predicted standards. This is in general accord with the results of Martin,[12] Mason,[13] and Tilt and Walters,[14] obtained upon subjects in tropical or subtropical climates.

SUMMARY

1. The apparent basal metabolic rate after smoking generally shows an appreciable deviation from a control rate determined prior to smoking.

2. The generally accepted rise in basal metabolism some time after smoking is by no means a universal phenomenon.

3. A rise in pulse rate, respiration rate, and body temperature following smoking is generally observed.

4. The sex of the subject apparently does not influence his energy output following smoking.

5. A possible explanation of the individual response to smoking is offered.

Grateful acknowledgment is made to our voluntary subjects for their faithful cooperation, and to Dr. William C. Boeck, of Los Angeles, for his helpful suggestion with regard to interpretation of our data.

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LABORATORY METHODS

GENERAL

SLIDE AGGLUTINATION IN DETECTING PERTUSSIS ANTIBODIES*

Comparison of Slide With Tube Agglutination

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DIAGNOSIS by means of slide agglutination has been used successfully for some time in Brucellosis,[1-6] and in typhoid-paratyphoid infections.[7, 8] More recently Evans and Maitland[9] recommended slide agglutination for the diagnosis of pertussis. Using a concentrated antigen of live *H. pertussis* and examining the slides microscopically, they obtained more positive results on the slide than on macroscopic tube agglutination.

The main advantages of slide over tube agglutination are (1) the small amount of serum required for the test, (2) the rapidity of the reaction (agglutination usually occurs within a few minutes) and (3) the simple apparatus necessary to carry out the test. Therefore, if on further tests slide agglutination should prove sufficiently sensitive to detect weakly positive sera, it would be an improvement on the more cumbersome and time-consuming method of tube agglutination. With this object in view we have tested a fairly large number of sera by slide and tube agglutination.

The use of a live antigen in slide agglutination as recommended by Evans and Maitland[9] seemed undesirable, since it would be necessary to prepare a suspension of the bacilli and standardize it for each day's tests. We found, after several trials, that we could use with equal success a suspension that was killed by means of 0.5 per cent formalin, or 1:50,000 dilution of merthiolate. This antigen did not lose its agglutinability after seven months' storage, which was the period of this investigation. Antigens that were killed by means of gentian violet in a 1:20,000 dilution or brilliant green in a 1:40,000 dilution became less agglutinable upon storage and were therefore not used. The antigen that we employed in our tests was prepared as follows:

A forty-eight-hour growth of *H. pertussis* on freshly prepared Bordet-Gengou medium was emulsified in sterile physiologic saline. The suspension was filtered through sterile cotton to remove particles of agar, and the bacteria were precipitated by centrifugation. They were washed twice in saline, and after the second washing they were suspended in physiologic saline which contained 1:50,000 merthiolate. The antigen was stored at 8 to 10° C.

The optimum concentration of the antigen for rapid slide agglutination was determined by titration with an antipertussis rabbit serum and was later

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checked with known positive human sera. Serial dilutions were made of the positive serum, and each dilution was tested with various concentrations of the antigen. As a control a live suspension of the bacilli was used in a concentration of 100 billion as recommended by Evans and Maitland. We found that a concentration of 25 billion bacilli per milliliter of the killed antigen gave more clear-cut agglutination than higher concentrations, and agglutination took place in the same dilutions as with the live antigen. We therefore used our antigen in a 25 billion concentration.

Before each day's test, the antigen was diluted with sterile physiologic saline to 25 billion bacilli per milliliter and was filtered through a 60-mesh monel metal wire gauze to remove clumps. It was then titrated with the positive serum to determine its sensitivity on the day of the test. The same positive serum was used throughout this study, and its titer remained remarkably constant.

The Test.—The sera were tested undiluted; 0.01, 0.02, 0.04, and 0.08 ml. amounts were used according to the method of Welch-Stuart[7] for typhoid-paratyphoid diagnosis. The stated amounts of serum were measured into the center of 4 paraffin rings on a glass slide by means of a pipette that was graduated into 0.01 ml. A drop of the antigen was added to the serum from a standardized capillary pipette that delivered approximately 0.015 ml. in each drop. The serum and antigen were thoroughly mixed by means of a wooden applicator, beginning with the smallest amount of serum. The slide was tilted back and forth for one minute, and a preliminary reading was made. The final reading was made after an additional three minutes' incubation at room temperature. All readings were made macroscopically, using a constant light. We found it unnecessary to read the agglutination on the slide microscopically since the clumps were distinctly visible to the naked eye.

Strongly positive sera agglutinated usually within one minute; weakly positive sera required two to three minutes. Where agglutination was complete, the clumps were large and the fluid clear. A serum was considered negative when it showed incomplete or no agglutination after four minutes' incubation at room temperature; a longer period of incubation could not be used, as the mixtures began to dry after four minutes.

Agglutination usually took place first in the 0.01 and 0.02 ml. then in the 0.04 and 0.08 ml. amounts of the serum, thus showing that the optimum proportion of serum to antigen was in the 0.01 or 0.02 ml. amounts of the serum. The final results were recorded on the basis of agglutination in 0.01 ml. of the serum. When agglutination was complete in the undiluted serum, serial dilutions were made, and 0.01 ml. of each dilution was tested for agglutination. The result of the titration was recorded in terms of the highest dilution of the serum that showed distinctly visible clumps.

TUBE AGGLUTINATION

Antigen.—The preparation of the antigen was described in detail in a previous publication.[10] A formalinized saline suspension of a forty-eight-hour growth of *H. pertussis* on Bordet-Gengou medium was standardized and was tested for agglutination with a known positive serum. It was stored in con-

TABLE I

SLIDE VERSUS TUBE AGGLUTINATION IN DETECTING PERTUSSIS ANTIBODIES

HISTORY	TOTAL SERA	AGGLUTINATION			TOTAL POSITIVE	
		NO.	SLIDE	TUBE	SLIDE	TUBE
Persons vaccinated against pertussis	19	19	+	+	19	19
Mice vaccinated with various pertussis antigens	23*	16	+	+	19	16
		3	+	0		
		4	0	0		
Normal mice	3†	3	0	0	0	0
Persons with history of whooping cough	30	2	+	+	8	2
		6	+	0		
		22	0	0		
Persons with colds or coughs definitely exposed to whooping cough	50	6	+	+	14	7
		8	+	0		
		1	0	+		
		35	0	0		
Persons without clinical symptoms definitely exposed to whooping cough	6	2	+	+	2	2
		4	0	0		
Persons with colds or coughs probably exposed to whooping cough	81	7	+	+	14	9
		7	+	0		
		2	0	+		
		65	0	0		
Persons with no illness probably exposed to whooping cough	2	2	0	0	0	0
Persons with colds and no known exposures to whooping cough	45	2	+	+	2	2
		43	0	0		
Persons without illness and no known exposures to whooping cough	72	3	+	+	3	4
		1	0	+		
		68	0	0		
Miscellaneous sera for pertussis diagnosis	11	2	+	+	6	3
		1	0	+		
		4	+	0		
		4	0	0		
Total no. of sera	342				87	64
Total positive sera	92					

*Each specimen was pooled from a group of mice vaccinated with the same antigen.
†Each serum represents a group of normal mice.

TABLE II

PERTUSSIS SLIDE VERSUS TUBE AGGLUTINATION

(Comparison of Agglutination Titers on the Slide and in the Tube)

NO. OF SERA	AGGLUTINATION TITER		REMARKS
	SLIDE	TUBE	
11	1:300-1:1,024	1:1,600-1:9,600	
6	1:64 -1:256	1:300 -1:2,400	
13	1:16 -1:40	1:50 -1:6,400	
10	1:5 -1:10	1:25 -1:400	
10	1:2 -1:4	1:10 -1:75	The agglutination titer is given in terms of the highest dilution that showed definite clumps
9	1:1	1:10 -1:150	
14	1:2 -1:8	0	
14	1:1	0	
5	0	1:5 -1:37.5	

Total number positive, 92 sera; positive on slide and tube agglutination, 59 sera; positive on slide only, 28 sera; positive on tube only, 5 sera.

centrated form at 8 to 10° C. On the day of the test the antigen was diluted to contain approximately 2,500 million bacilli per milliliter.

The Test.—The sera were tested in 1:10 to 1:1,000 dilution or higher if necessary. To 0.1 ml. of the serum dilutions, 0.9 ml. of the antigen was added, and the tests were incubated in a water bath overnight at 52° to 56° C. An antigen control in saline without serum, and an antigen titration with a positive serum were included in each test. Readings were made macroscopically, using a constant light. The titer of the serum was recorded in terms of the highest dilution that showed distinctly visible clumps. For the purpose of comparison with slide agglutination, a serum was recorded positive on tube agglutination when there was distinct agglutination in the 1:10 dilution of the serum. Doubtful or no agglutination in the 1:10 dilution when all the higher dilutions were negative was recorded as negative.

We tested 342 sera for slide and tube agglutination. Among these were sera from persons who were vaccinated against pertussis; from groups of mice that were immunized with various pertussis antigens; from persons with clinical pertussis; from persons with colds or coughs, who were exposed to pertussis; from persons without illness some of whom had been exposed to pertussis; and from groups of normal mice. The results are summarized in Tables I and II.

Table I shows that more positive results were obtained on slide than on tube agglutination with sera of persons who had clinical pertussis and those who were suspected of having pertussis, or were exposed to actual cases of this disease. Slide agglutination appears to be specific since no more positive results were obtained on the slide than on tube agglutination among persons who were not exposed to pertussis, although a large number of them had coughs or head colds at the time of the test.

Table II shows that the agglutination titers on the slide and in the tube did not run parallel. On the whole however, all of the sera that had a high titer on tube agglutination also had a fairly high titer on slide agglutination. Slide agglutination proved to be more sensitive than tube agglutination in detecting weakly positive sera as there were 28 sera positive on the slide and negative on tube agglutinations, as compared with 5 sera that gave a low titer of agglutination in the tube and were negative on the slide.

CONCLUSION

1. The results obtained in this study have confirmed the findings of Evans and Maitland that more positives are obtained on slide than on tube agglutination in detecting pertussis antibodies.

2. We found that an antigen that was preserved with merthiolate and stored at 8 to 10° C. had given equally good results as a live antigen that was recommended by Evans and Maitland, and it retained its agglutinability during the seven months' period of this study. In all probability it will remain unchanged for a much longer time. This antigen has the advantage over a live antigen in that it is always available and it eliminates the necessity of preparing and standardizing the antigen for each day's test.

3. The results obtained on slide agglutination were as specific as on tube agglutination since no more positives were obtained on the slide than on tube agglutination with sera from persons who had no clinical symptoms of pertussis and no history of exposure to pertussis.

4. Slide agglutination has the advantage over tube agglutination in that the result may be obtained within a few minutes, and with a carefully standardized antigen more positive results may be obtained with weakly positive sera.

5. In the testing of large numbers of sera, slide agglutination would be very helpful in eliminating negative or doubtful sera. The positive sera could then be titrated by tube agglutination if the exact titer of the serum is required.

6. From present indications a positive result on slide agglutination may be taken as diagnostic of pertussis infection, when there was no previous pertussis vaccination and no vaccine therapy. A negative result does not exclude pertussis infection since a fairly large number of clinically typical pertussis patients do not develop agglutinins until late in the disease or until convalescence.

Technical assistance in this study was given by Edna Lewis and Ruth Orange in carrying out the tube agglutination tests.

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A SIMPLE DEVICE FOR THE PROTECTION OF THERMOSTATS IN WATER BATHS*

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IN MANY electrically operated water baths, the thermostatic control necessary to maintain a constant temperature is exposed to the destructive action of mercury if the thermometer is broken.

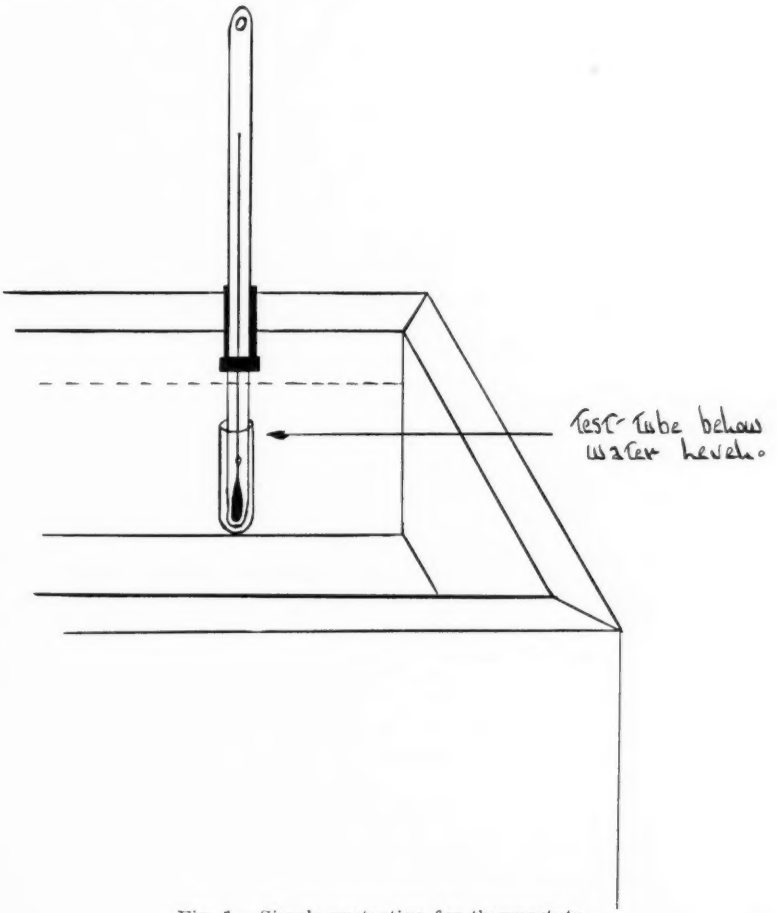

Fig. 1.—Simple protection for thermostats.

Mercury can ruin a water bath beyond repair by attacking the solder of joints in the bath and the metals in the thermostat. This can be avoided by placing a small test tube around the bulb of the thermometer in such a manner that the top of the tube is below the water level. The test tube can be adjusted to fit the type of water bath and thermometer as required. In the event of thermometer breakage, the mercury will fall into the test tube. A simple clamp device that will hold the tube upright can be constructed, depending upon the type of water bath. The mercury therefore will not come in contact with metal connections.

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THE ANTIFOGGING EFFECT OF SOAP ON EYEGLASSES AND DIAGNOSTIC MIRRORS*

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TO ANY physician who has had to operate wearing eyeglasses and a face mask, fogging or steaming of the glasses is a real hardship. The mask diverts the warm exhaled air along each side of the nose and causes it to condense on the cooler surfaces of the eyeglass lenses. Fogging results, and this makes it difficult for him to see through the lenses.

Johnson[1] discussed this problem in a short article and contributed a unique solution. He attached a piece of adhesive tape along the top of the mask and pasted this to the skin of the nose. This does not allow the warm air coming from the mouth and nose to be diverted toward the glasses. Other contrivances, such as metallic inserts along the top of the face mask and loosely fitting sacs covering the whole head, have also been tried, but these require special equipment.

The present contribution concerns means of treating the glasses themselves so that the warm air cannot condense on their surfaces. It consists of chalking a few lines across the lens with a piece of soap, and then polishing the glass with soft paper or a handkerchief. A thin coating of soap remains on the surface which repels the condensation of the warm air. The visibility is not decreased by this fine coating.

The method is also applicable to laryngeal mirrors and other diagnostic mirrors that come in contact with warm, moist air. Other methods, such as warming the mirror or coating it with saliva, are in use, but the simplicity of the present plan serves to recommend it. For diagnostic mirrors, I have found the antifogging effect of naphtha soap to be more effective than ordinary white soap.

Realizing that a solution so simple could hardly be original, I referred to a book[2] published by Henley and Company, which contains among its 10,000 formulas, one which prevents the "dimming" of eyeglasses and other glassware. The formula consists of adding a form of soap to glycerin and oil of turpentine.

SUMMARY

Soap exerts an antifogging action on eyeglasses and diagnostic mirrors.

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A SIMPLIFIED "LYOPHILE" DESICCATOR FOR SMALL LABORATORIES*

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A NUMBER of devices have been described[1-5] for the desiccation of biological materials from the frozen state by evaporation of the water under a high vacuum, with subsequent condensation of the vapor in a condenser held at a very low temperature. For this process, the term "lyophile" has been proposed.[1, 3] These devices have been intended primarily for the processing of relatively large volumes, and as a consequence have a much greater capacity than is necessary for the handling of occasional small batches of material. Further, these devices are in general of a somewhat complicated design, necessitating the services of a machine shop and skilled workmen in their construction. It is the purpose of this paper to describe a modification of the lyophile apparatus, developed for use in a small laboratory, which possesses certain advantages with regard to convenience, adaptability, simplicity of construction, and inexpensiveness.

Fig. 1 presents a diagram of the apparatus. *A* is the condensing unit, a 1 liter, short neck, pyrex flask, fitted with a 34/45 standard taper ground glass joint and a 3-way stopcock of large bore, *C*. *B*, the connection between the condenser and the vessel containing the material to be dried, *F*, is made of pyrex tubing with an internal diameter of approximately ¾ inch, bent at right angles at each end, and fitted with two 34/45 standard taper ground joints (inner part) as illustrated. The end fitting the condenser is extended beyond the ground joint to within 1½ inches of the bottom of the flask. *D* is a large metal can holding the freezing mixture of dry ice and 95 per cent alcohol; it in turn is held in a wooden box *E* surrounded by 2 inches of fine sawdust or other suitable insulating material.

In use, a mixture of cracked dry ice and alcohol is placed in the metal container, and the condensing flask is immersed to thoroughly chill it. Five pounds of dry ice will operate the apparatus for six hours or more. The alcohol may be re-used (usually about six times) until it has absorbed enough water from the atmosphere so that ice crystals form in it when the dry ice is added. The material to be dried is frozen around the interior of a suitable container by rotating the container and contents for a few moments in the dry ice-alcohol bath. The rate of drying is dependent in a large measure on the total surface area of the material to be dried, so that the layer of frozen material is made as thin as practical. The apparatus is then assembled as illustrated, the joints being made vacuum tight by means of a suitable sealing compound such as "Lubriseal." Evacuation of the apparatus is carried out by means of a Ceneo

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Hyvac pump, or its equivalent, attached to the three-way stopcock during the entire period of desiccation. A McCleod gauge is not essential to determine the degree of vacuum. In our experience the pump is operating satisfactorily if the valves make a sharp metallic click as they seat. It has not been found necessary to interpose a trap between the condenser and the pump to prevent water vapor from entering the pump, as suggested by several workers.[2, 3, 5] In this laboratory the oil in the pump is changed every four to six months, and while it contains some water, it does not seem to affect materially the operation of the pump. In this connection, removal of the water and debris from the oil by centrifugation in a Sharples centrifuge renders it again fit for use. When desiccation of the material is complete, the vacuum is broken by turning the three-way cock, and the material is removed from the desiccator.

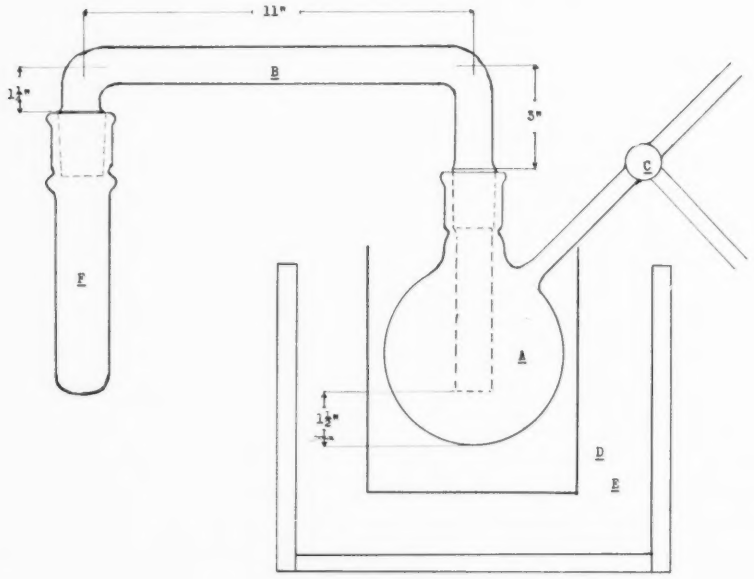

Fig. 1.

By virtue of the standard taper joints it is possible to attach a wide variety of desiccation vessels to the apparatus. For desiccation of small volumes of material, a container made by sealing the open end of a 34/45 standard taper pyrex ground joint (outer part) has proved very satisfactory (Fig. 1, *F*). The most generally useful desiccation vessel in this laboratory has been a small vacuum desiccator (140 mm. inside diameter) with a ground joint in the lid fitting the pyrex 34/45 ground joint. It is not necessary to have this lid made to special order, since it was found after testing several sizes and makes of desiccators that several are made with a ground joint fitting perfectly with the pyrex standard taper joint. Corning Glass Works also manufacture a large pyrex vacuum desiccator, which is equipped with a 34/45 standard taper joint in the lid. In using either the small or large desiccator, the material to be dried is frozen around the sides of one or more beakers or other suitable containers, placed in the desiccator, and the desiccator is attached to the condensing unit.

Evaporation of the water vapor proceeds at a sufficiently rapid rate so that the material to be dried remains frozen during the entire period of desiccation. As shown by Greaves and Adair,[4] sublimation of water vapor can be materially increased by raising the temperature, taking care, of course, to avoid melting the frozen material. In using a flask as a desiccating vessel, this warming may be accomplished by immersing the flask in a water bath at 30° to 40° C. At such temperatures the material will remain frozen if the pump is operating satisfactorily. The vacuum desiccators may be wrapped with rubber tubing held in place with adhesive tape. After a high vacuum has been attained, warm water may be circulated through this tubing. It is necessary to use a somewhat higher temperature here (40° to 50° C.), since the vacuum within the desiccator insulates the contents to a certain extent. It is impossible to give figures on the rate of drying, since this rate is dependent on so many factors: the material to be dried, surface area of the material, degree of vacuum, temperature of the desiccation vessel, and the like. However, when drying antisera, frozen around the sides of 600 ml. beakers, a rate of about 15 to 20 c.c. per hour was observed.

The chief advantage of this modification of the lyophile apparatus is its versatility. In addition to the desiccation vessels mentioned, there are in use in this laboratory several sizes and shapes of flasks and a pyrex manifold for the desiccation of small amounts of material in tubes with subsequent sealing of the tubes under vacuum. Other advantages of this apparatus are its simplicity and economy. A moderately skilled glass blower should be able to construct it from standard items of pyrex glassware in a few hours, or the Corning Glass Works will manufacture the apparatus to specifications for a reasonable sum. By no means least of the advantages is the ease and simplicity of cleaning and removal of frozen water vapor. It is necessary merely to disconnect the condenser from the connecting tube, thaw under the tap, and empty. This operation may be performed rapidly enough so that the material in the desiccation vessel does not thaw.

This apparatus has been used in this laboratory primarily for the desiccation of partially purified fibrinogen and prothrombin preparations to be used in the study of streptococcal fibrinolysin.[6] The precipitated material, either fibrinogen or prothrombin, is dissolved in a small amount of water, frozen, and dried. It has been found that desiccation in the lyophile apparatus instead of over phosphorous pentoxide, as originally recommended,[7, 8] results in greater solubility, less denaturation, and greater stability of these materials. In addition to fibrinogen and prothrombin, materials dried in this apparatus have included partially purified fibrinolysin, bacterial polysaccharides, cultures of various organisms, virus suspensions, plasma and serum, and various antisera. Other possible uses include preservation of complement, typing sera, and soluble antigens.

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SIMPLE MODIFICATION OF THE HANIKE-GIBBS DROP RECORDER*

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THE Hanike-Gibbs instrument for recording drops of secretion applies the principle of displacement of an electrolyte solution with constant viscosity and conductivity. The instrument reported by Gibbs[1] in 1927 consists of 5 different parts with a number of connections and does not lend itself easily to experiments in which the rates of a number of different secretions must be measured at the same time. We have therefore modified the instrument, using the same principle.

Fig. 1 is a diagram of the modified drop recorder. The upper part of two glass cylinders *A* and *B* is solidly connected by a glass tube *C*. The first cylinder *A* is empty and connected to the cannulated duct of an organ, the secretion of which is to be measured. The second cylinder *B* is filled with a 10 per cent solution of sodium citrate.† The lower pole of this cylinder has a fine opening *F* through which a drop of the citrate solution is forced when a drop of secretion displaces air in the first cylinder. The falling citrate drop passes between platinum wire *K* and the brass funnel *L*, fixed in a glass tube *G* to which slight constant water suction is applied so that the drop of citrate passes rapidly between the contacts. The drop closes the electric circuit temporarily while touching the two contacts, and the signal magnet is activated. The contacts are connected from binding posts *H* and *J* in series with 115 volts A.C. or D.C. and to a small recording signal magnet. A fuse is introduced into the circuit. Due to the constant suction the amount of current transmitted is small enough to permit the use of an ordinary 6-volt laboratory signal magnet. The instrument can be made in the laboratory at low cost. We have used it for more than two years and found it so reliable, easy to use, and maintain that we feel it would be useful to many other laboratories. The instrument must be protected from radiating heat which will expand the air and citrate solution in the two chambers. The instrument will work for many hours unsupervised.

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†Lately we have used instead a 3 per cent solution of commercial sulfuric acid, which is cheaper and which keeps the contacts *K* and *L* clean.

Samples of secretion can be removed from *E* at intervals for analysis. The samples are not diluted or mixed wth other substances, and they are not exposed to electric sparks and electrolysis as in some other instruments. Refilling can be done swiftly and easily by turning the current off (a switch can be inserted at *J*), opening the clamps at *E* and *D*, and attaching a funnel to the

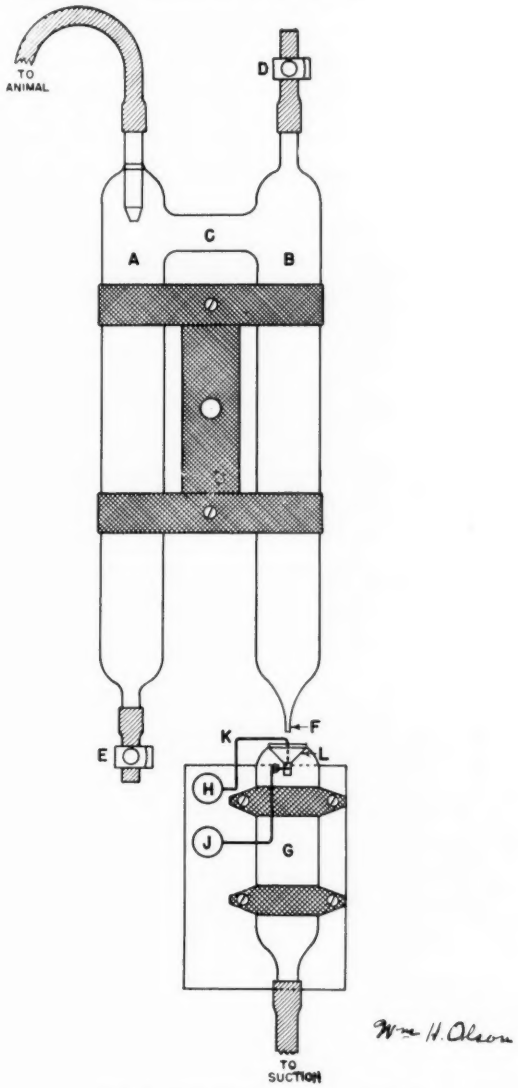

Fig. 1.—Diagram of drop recorder.

rubber tube at *D*. After *B* has been filled with the citrate solution to just below the level of *C*, clamp *D* is closed quickly. During the process of filling, some of the citrate solution escapes through *F* which may be closed during the filling, but we have not found that necessary. It may take then a few minutes before pressure equilibrium is reached in the system. We have found it of advantage to put the drop recorder below the level of the cannulated duct of the animal, because a slight suction on the duct may prevent clogging or, in the case of lymph, clotting.

We have used a set of 4 instruments, as shown in Fig. 2, to record simultaneously salivary, pancreatic, biliary, and lymph secretion.

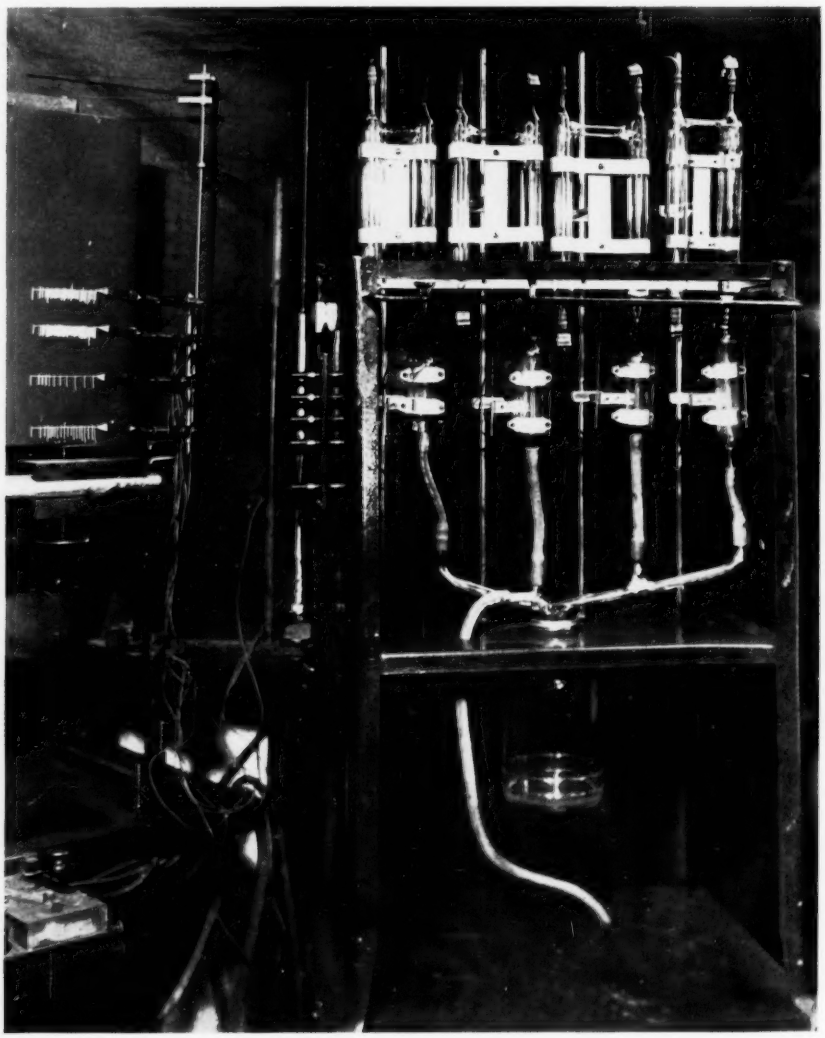

Fig. 2.

DISCUSSION

We feel that, when a continuous quantitative record of the volume of a secretion is wanted, the displacement system should be employed in order to obtain more accurate results. For example, in the measuring of the secretion of bile or saliva, the concentrations of salts and of mucus usually change during the experiment. This results in a change in the viscosity of the secretion and in the size of the individual drop. Thus we have often found the size of a drop of bile to double its volume as the experiment progressed. Such uncontrollable changes can be avoided when a solution of constant composition is displaced by the secretion.

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A SATISFACTORY TECHNIQUE FOR DELAYED TYPING OF THE PNEUMOCOCCUS*

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WITH the widespread immediate use of the sulfa drugs instead of serum in the treatment of pneumonia, the typing of the pneumococcus seems of little value except for case records. Laboratory directions always state that a sputum is best typed as soon as possible after collection because of the early death of the pneumococcus and the overgrowth of contaminating bacteria. This work is presented to show that this is unnecessary except when "stat" typings are desired.

The suggested technique is based on the following work. Typings were run on more than 50 sputa of known type. Three generous smears were made on each of 6 glass slides and allowed to dry in the air. They were then wrapped in paper and stored, 3 at room temperature and 3 at icebox temperature. After intervals of twenty-four, forty-eight, and seventy-two hours, and one, two, three, and four weeks, each type was tested for the quellung reaction in the ordinary manner. It was found that all slides stored at either temperature for the varying lengths of time typed just as well as the original specimen. A few slides tested after storage for ten months gave good quellung reactions. There was no apparent reduction in the number of swellings or the size of the swollen capsule. Group sera swelled stored preparations as well as the type sera. To check against high temperatures of the tropics several types when dried were stored at incubator temperature (37.5° C.) for one month. These still gave good swellings. All types have been tested except types IX, XI, XXVII, XXVIII, XXXI, and XXXII which were not encountered during the period when the work was being done.

As a result of this work, it is suggested that exudates (with a few exceptions such as spinal fluids requiring centrifugation) immediately upon collection be smeared entirely across or at three locations on each of at least four clean slides and allowed to dry in the air. These may then be typed at one's convenience in the usual manner.

Since this seems to prove that delayed typings are possible, several advantages can be observed in the use of dried slides for typings:

1. Dry fields are easier to examine microscopically since there is less motion present than in a moist smear.

2. Drying on a slide keeps the organisms from deteriorating and contaminating bacteria from overgrowing and destroying the pneumococci.

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3. Dried slides can easily be mailed great distances for typing, and variance in time would not alter the results.

4. Smears from the pharynx, especially in children, can be made immediately and typed easily by this method without the risk of the swab's drying before it can be typed.

5. The most important advantage from the technician's standpoint is the lack of the necessity of "stat" work.

TRYPSIN AS A DIGESTANT OF SPUTUM AND OTHER BODY FLUIDS PRELIMINARY TO EXAMINATION FOR ACID-FAST BACILLI*

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A NUMBER of methods have been used for the concentration of sputum preliminary to examination for acid-fast bacilli. Some of these fail to concentrate the organisms adequately; others are time consuming; and some require special apparatus and delicate technique. Since 1933 we have used an aqueous solution of trypsin to digest such specimens as sputum, stomach washings, pleural and abdominal fluids, urine that contained mucus, and pus from abscesses, with satisfactory results.

METHOD

A 0.5 per cent trypsin solution is made by adding powdered trypsin† 1:250 (Difco) to sterile water. Mix by shaking, and add 0.7 per cent N/1 sodium hydroxide solution, which gives approximately pH 8.5. Since trypsin acts over a wide range of hydrogen-ion concentration, it is not absolutely essential that the sodium hydroxide be added, but the trypsin is most active at pH 8.0 to 8.5. The trypsin solution should be kept in the refrigerator or, better, filtered through a Seitz filter if it is not used shortly after it is prepared.

1. An equal quantity of the trypsin solution, or more if the specimen is very thick, is added to the specimen in a bottle or large test tube, and mixed thoroughly, an applicator stick or glass rod being useful for this purpose.

2. The specimen is then incubated at 37° C. for thirty minutes, or until the mucus is thoroughly digested, which can be determined by drawing out the applicator stick or by the sedimentation of the cells. Stirring after ten to fifteen minutes' incubation may hasten the digestion.

3. After digestion is complete, the specimen is centrifuged at 3,000 r.p.m. for five minutes, or at 1,500 r.p.m. for ten minutes.

4. A loopful of the sediment is smeared on a glass slide, fixed as usual, and stained by the Ziehl-Neelsen method. If the smear is thin, methylene blue is used as a counterstain; if it is thick, 1 per cent aqueous picric acid is used. The sediment is also satisfactory for examination by the fluorescence method.

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†Difco Laboratories, Inc., Detroit, Mich.

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EXPERIMENTAL DATA

In order to compare the effectiveness of concentration of acid-fast bacilli in sputum by the trypsin method with the flocculation method of Hanks et al.,[1] a series of specimens was examined by both methods. Preliminary to digestion the specimens were heated in an Arnold sterilizer for thirty minutes and cooled (this is done routinely in our laboratory as a safety measure unless the specimen is to be used for culture or guinea pig inoculation). The specimens were then divided as evenly as possible (we used as much as 5 c.c. if the size of the specimen permitted) and put into 6 inch by ¾ inch test tubes. The procedure of Hanks et al.[1] for the concentration of tubercle bacilli and the trypsin method as outlined above were followed. Smears were made in a uniform manner, a 4 mm. loopful of the sediment being spread over an area 1.5 cm. by 3.5 cm., and fixed and stained by the Ziehl-Neelsen method. The acid-fast bacilli in 200 fields were counted, the number of fields being recorded as the bacilli were counted. Since the finding of a clump of bacilli does not increase the possibilities of finding the organism, each clump was counted as one.

Of 100 specimens of sputum which were positive by one or both methods, 14 per cent were positive by the trypsin method that were negative by the flocculation technique and 3 per cent were negative by the trypsin method which were positive by the flocculation technique. In 5 per cent of the specimens the same number of bacilli were found by both methods. In the remaining 95 specimens more acid-fast bacilli were found in 71.5 per cent of the trypsin-digested specimens, and in 30 per cent of these at least twice as many acid-fast bacilli were found in 200 fields by the trypsin technique as by the flocculation method. More acid-fast bacilli were found in 28.4 per cent of the specimens by the flocculation method, and 7 per cent of these had at least twice as many acid-fast bacilli as were found by the trypsin method.

DISCUSSION

As the results show, 11 per cent more specimens were positive by the trypsin method than by the flocculation method. Also more acid-fast bacilli were found in 71.5 per cent of the trypsin-digested specimens in contrast to 28.4 per cent of the specimens which showed more acid-fast bacilli in the portion of the specimens concentrated by the flocculation method. This indicates that a specimen might be found positive by both methods, but the bacilli would probably be found in less time in smears made from the trypsin-digested specimen. This is indicated further by the fact that 30 per cent of the smears made from the trypsin-digested specimen showed at least twice as many bacilli as the same specimen concentrated by the flocculation technique, while, of those smears which gave a higher count by the flocculation method, only 7 per cent had at least twice as many of the bacilli as were found by the trypsin method.

Not only is there a reduction in the time required for examination of smears over that required by some other methods, but the time necessary to carry out the procedure of digestion and concentration is reduced to a minimum. The steps of the procedure are few and simple. No delicate or involved technique is necessary, and no special apparatus is required. Although sputa vary considerably in hydrogen-ion concentration (from pH 5.0 to pH 7.5), it is not necessary to adjust each specimen to a certain hydrogen-ion concentration since trypsin is active over a wide range.

Sedimentation always seems to be very complete after thorough digestion of the mucus. The specific gravity is low (around 1.006 to 1.011), and the surface tension is such that it does not interfere with complete sedimentation. Recentrifugation of the supernatant fluid from a few positive specimens gave very little or no sediment and no positive smears.

Digestion of sputum which has been heated proceeds faster than that of unheated sputum if it contains considerable mucus. After heating, specimens may contain lumps of coagulated material, but these are soft and easily broken by means of a applicator stick or glass rod.

Considerable sediment is obtained from trypsin-digested specimens as the other organisms and body cells present are not digested. This we consider an advantage as they give a background which enables the technician to keep the field in focus more easily. Also any other organisms that may be of clinical importance, such as those of Vincent's infection and fungi, may be seen. If the smear is thick, however, picric acid is preferred as a counterstain, for methylene blue may stain so heavily that the acid-fast bacilli cannot be seen. If picric acid is used for the counter stain, a smear can be stained by another method for other organisms. The sediment is usually creamy in consistency and spreads easily, and the smears stick well to the slide.

The presence of phenol in specimens of sputum does not interfere with digestion by trypsin. Specimens in which unknown amounts of phenol were present have been digested with trypsin solution with no difficulty. To one specimen an equal amount of 5 per cent phenol was added and to that mixture an equal amount of trypsin, giving 1.25 per cent of phenol. No inhibition of the enzymatic action was observed; on the contrary, it seemed to be stimulated. It might be stated that trypsin has been used for the digestion of sputum specimens by the Indiana State Board of Health for the past three years, with satisfaction, all of the specimens being collected in bottles which are from one-third to one-half full of 5 per cent phenol when issued from the laboratory.

For the past eight years we have also used trypsin to digest specimens preliminary to culturing or guinea pig inoculation. Acid-fast bacilli treated as long as twenty-four hours with trypsin solutions are viable and will infect a guinea pig. After digestion of the specimen with trypsin the sediment is treated with oxalic acid to kill the other organisms present according to Corper's method[2] for culture, or prepared for guinea pig inoculation.

The trypsin powder is not sterile, and bacteria and higher fungi will grow in a solution of it, even at low temperatures. As these might prove a source of error, sterilization by filtering through a Seitz filter is recommended. However, we have found that unfiltered trypsin solution, if made with aseptic precautions and kept in the refrigerator, will remain in good condition for a few days, but one must be alert to the possibilities of error when using it. Also, if the solution is used immediately after preparation for the digestion of specimens for smears, it is not necessary to filter it. To digest specimens for culturing or guinea pig inoculation, sterile trypsin solution should be used.

Trypsin powder is relatively inexpensive. The amount needed for the digestion of a specimen costs less than one cent.

SUMMARY

A method of digesting sputum and other body fluids with trypsin preliminary to microscopic examination, culturing and guinea pig inoculation, has been presented.

This method is considered to be practical for it is simpler in procedure than other satisfactory methods and requires less of the technician's time.

In a comparative study of this method and the flocculation method of Hanks et al.,[1] of 100 positive specimens, 11 per cent more positives were found by the trypsin method. In 71.5 per cent of the smears made from the trypsin-digested portion of the specimens, more acid-fast bacilli were found than in smears made from the flocculation concentrate, and in 30 per cent of these at least twice as many bacilli were found. In contrast to these results, only 28.4 per cent of the smears made from the flocculation concentrate showed more bacilli than those made from the trypsin-digested portion of the specimen, and, of these, only 7 per cent showed twice as many or more acid-fast bacilli.

The author wishes to express gratitude to Dr. C. G. Culbertson and Dr. R. N. Harger for suggestions and encouragement, to Dr. Russell S. Henry, Mrs. R. C. Speas, and Mr. Frank E. Lane for supplying specimens from Sunnyside Sanatorium, and to Mrs. Martha Ann Deppe and Mr. George E. McCoy, who assisted with the experimental work.

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AN IMPROVISED INVERTED MICROSCOPE*[1]

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THE inverted microscope has been employed in photomicrography,[2, 3] in tissue culture,[4] and in micrurgy.[5] Special advantages accrue from its employment in these various fields, and in the field of micrurgy these advantages have been sufficient to warrant the appearance of a commercial instrument.[5]

A description is herein given of an improvised inverted microscope which possesses the advantages of being readily available and inexpensive, for, except for two small prisms, the instrument is assembled from (1) microscope parts commonly available in the laboratory and (2) a few special parts which can easily be fabricated. The microscope parts used in the assembly suffer no mutilation. The instrument is designed especially for employment in conjunction with the Taylor micromanipulator,[6] but no difficulty should attach to its alteration for employment in conjunction with the Chambers micromanipulator.[5]

Fig. 1 presents the essentials of the arrangement. The arm (*A*) and body tube (*B-T*) of a standard microscope are affixed to a cast-iron base (*B*). A prism-chamber (*p-c*) replaces the nosepiece, and supports the three standard

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objective lenses. A sturdy upright (*U*), mounted on the base (*B*), supports the condenser (*C*) and light source (*L*). A mechanical stage (*S*) is fixed to the base (*B*) and serves in turn as base for a thin upright (*u*) which supports, between the condenser (*C*) and the objective, a coverslip which serves as a "stage" (*s*).

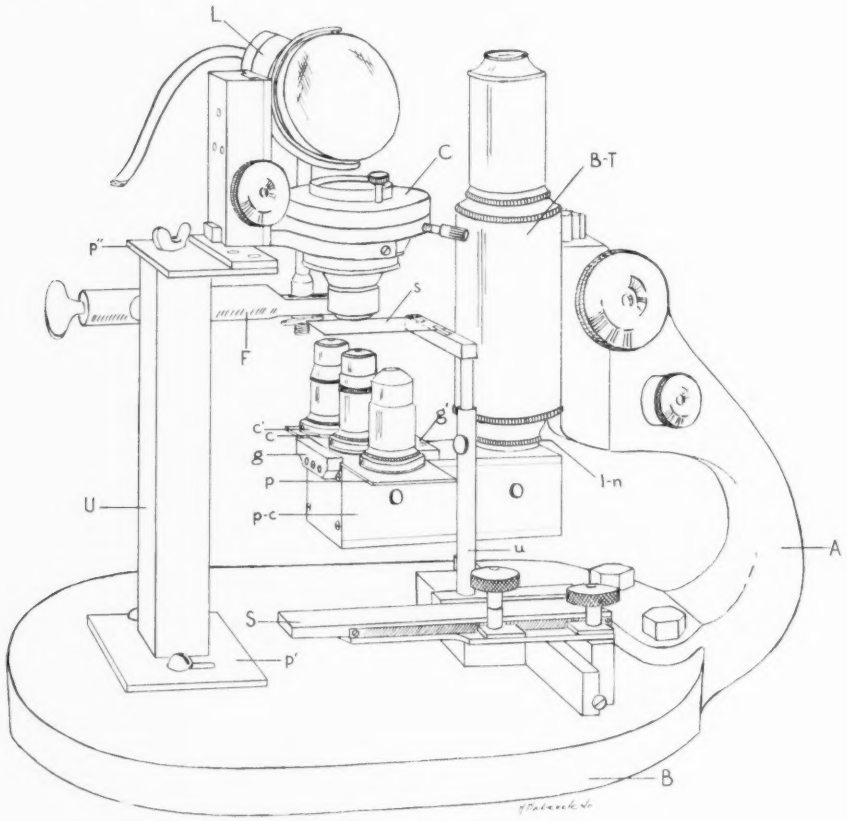

Fig. 1.

The following details concerning the various parts of the instrument are pertinent (see Figs. 1 and 2):

1. The arm (*A*) and body tube (*B-T*) are taken from a model 66 Spencer microscope.

2. The prism-chamber (*p-c*) is constructed of ⅛ inch sheet aluminum. It is 3¼ inches long, and its internal cross section measures 1 square inch. It is screwed into the body tube (*B-T*) by means of a threaded collar (*tc*), and is locked in proper position by means of a lock-nut (*l-n*). Two ⅜ inch holes are drilled in the roof of the prism-chamber (*p-c*), one concentric with the body tube (*B-T*) and the other concentric with the objective. Contained within the prism-chamber (*p-c*) are two right-angle prisms, as indicated in Fig. 2; these fit snugly into the chamber (*p-c*), and are held in position by means of screws (*x*) projecting through the floor of the chamber (*p-c*) and by plates which close its ends. The three objectives are mounted on a brass plate (*p*), each threaded into a collar (*c*), and this plate (*p*) is movable in a line perpendicular to the length of the prism-chamber (*p-c*). The plate (*p*) moves between two guides (*g, g'*).

3. A nicely-machined band (*c'*) fits snugly about the collar (*c*) into which the objective is threaded and can be fixed by means of a set-screw. Each band (*c'*) bears a small ma-

chined depression (not shown) which, when the desired objective is moved to center position, receives a spring-backed pin (not shown) projecting forward from the rear guide (g'). By means of these adjustable bands (c'), the objectives may be precisely centered.

4. The upright (U) which supports the condenser (C) and light source (L) is a 5½ inch length of ¾ inch square brass rod. A brass plate (p') fixed to its lower end serves for attachment to the base (B); this plate (p') has slotted screw-holes which allow for adjustment of position. A brass plate (p''), fixed to its upper end, supports the condenser (C); this plate (p'') is attached to the upright (U) by means of a centered screw, so that the condenser (C) can be swung aside readily.

5. The condenser (C) is an achromatic piece, of N.A. 1.3, as supplied for Model 3 Spencer research microscope. The entire assembly, including rack and pinion, is employed.

6. The light source (L) is an 18-volt toy train lamp. It is energized with 110-volt A.C. current, a variable rheostat being inserted in the line. The lamp housing is an objective case, in one end of which a frosted glass is mounted. This housing is supported on the upright (U) by means of an adjustable framework (F).

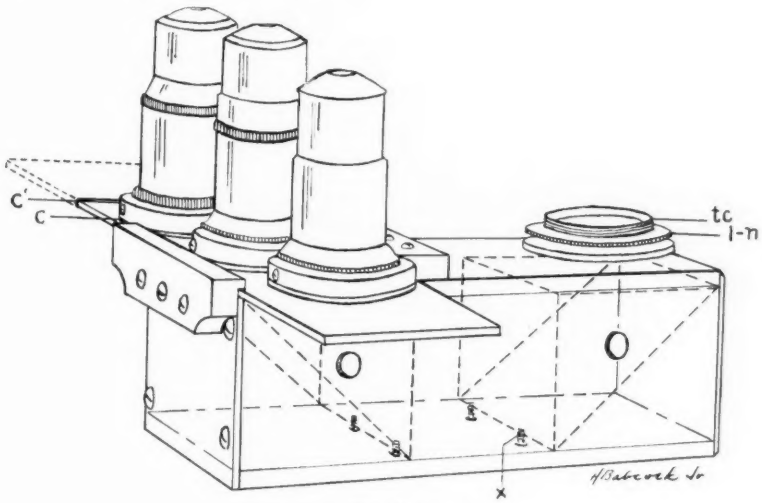

Fig. 2.

7. A Spencer attachable mechanical stage (S) is fixed to the base (B), slightly to the front and left of the arm (A). Its attachment is at right angles to its usual attachment, so that the movement which is normally lateral is here the front-back movement. Rising vertically from the front-back movement is the 5/16 inch circular upright (u), provided with a centered extension in the form of a 3/16 inch rod. A horizontal bar projects centrally from this extension, and supports the coverslip "stage" (s).

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CHEMICAL

DESICCATED PLASMA FOR NATIONAL DEFENSE*

Mass Production Methods Based on the Adtevac Process

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THE need of plasma in national defense is too urgent for prolonged consideration of theories and speculations. Extensive clinical results and proved production methods of large capacity are required to answer the following questions:

1. In what form is plasma most suitable for defense needs and military use?
2. Should plasma be administered in hypertonic, normal, or diluted form?
3. By what methods can mass production be achieved?

It is the purpose of this paper to answer these questions in the light of the experience of the desiccated plasma service of the William Buchanan Blood, Plasma, and Serum Center of Baylor University. This service during the past twenty-one months† has separated the plasma from 1,191,000 c.c. of blood, and desiccated plasma has been made available in Dallas and vicinity to the same extent as ordinary intravenous fluids. As a result, the value of this type of plasma used in hypertonic solution has been proved in extensive clinical trial.

On the basis of this experience the following answers to the three questions on use and preparation of plasma are given as follows:

1. For military as well as civilian use plasma desiccated from the frozen state is the ideal type of plasma preparation.

2. It should be used in hypertonic form (four times concentrated) to realize maximum advantages.

3. Mass production can be achieved with complete safety by the following principles: (a) Simplified collection, (b) Pooling whole bloods, (c) Separation of plasma by the continuous separator of the De Laval type, (d) Seitz filtration, (e) Bulk desiccation by the adtevac process, (f) Granulation and packaging of maximum quantity in minimum space in ordinary vaccine vials by a sterile mechanical dispenser, (g) Use of sterile room for open or semi-open phases of above methods, and (h) Bacteriologic control of all steps.

ADVANTAGES OF DESICCATED PLASMA

Desiccation from the frozen state offers advantages in the storage, transportation, and use of plasma that can scarcely be challenged. First, quality

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must be considered. The remarkable ability of this method of desiccation to preserve proteins and all biologic properties without change has been pointed out by workers in this field.[1]

Preservation of the proteins without deterioration is essential to assure solubility and to eliminate the possibility of protein denaturation as a cause of reactions. The excellent preservation of proteins by desiccation from the frozen state (sublimation desiccation) has been conclusively demonstrated by the electrophoretic studies of Scudder[2] and by our own clinical results.[3-6] For military use solubility should be measured in seconds rather than minutes, so that resolution may be done just prior to administration whether it be on the field, in the collecting station, in the aerial ambulance, or under whatever conditions encountered in peace or war.

Such rapid solubility has been consistently maintained in the Baylor service and has proved to be one of the chief advantages of the desiccated form over our earlier method of storing concentrated plasma in the frozen state. Solubilities average from thirty to 120 seconds. In actual treatment of shock, 25 Gm. of plasma have been dissolved in 90 c.c. of water and administered intravenously, all within 180 seconds. These rapid solubilities are maintained during storage, apparently depending on the very low moisture contents of 1 per cent and less, attainable by this type of desiccation.

Another advantage of dry plasma which has not been properly appreciated and utilized is the fact that it will not support bacterial growth. In this respect the plasma may be considered as any inert chemical powder. This property is of tremendous advantage for safety in use, for storage, and for mass production.

Contrary to the statement of Blalock and Mason,[7] a very definite advantage of reduced bulk is realized for desiccated plasma when packaged as described herein. For example, we placed amounts of plasma and water in two 60 c.c. vials, which is equivalent to 200 c.c. of normal plasma. This becomes a real advantage when the equipment for administration is included as discussed below.

The use of desiccated plasma has been considered expensive[8] and freezing proposed as an alternative method of preservation. From earlier extensive experience we have found that the routine preservation of plasma by freezing had many disadvantages which were eliminated by preservation in dry form. Low temperature refrigeration is costly and may well exceed the small cost of desiccation. When the extra safety and elimination of waste through spoiling or outdating are considered, desiccated plasma becomes definitely more economical. Furthermore, the problem of fibrin precipitation during storage of liquid plasma or upon thawing of frozen plasma is largely eliminated by desiccation. In the dry form fibrin precipitation is impossible and after resolution there is no increased tendency toward fibrin formation.

HYPERTONIC PLASMA, THE IDEAL FORM FOR ADMINISTRATION

One of the greatest advantages of desiccated plasma is that it provides the most logical way to make concentrated plasma solution. The ideal form in which to give plasma, particularly for the treatment of shock, is the four times concentrated plasma immediately after redissolving in pyrogen-free water.

Numerous advantages, both technical and therapeutic, realized by giving this hypertonic plasma are (1) increased simplicity and speed of administration with a glass syringe replacing more complicated apparatus, (2) elimination of tubing and the work of preparing it, (3) reduction of bulk and weight through decrease in water carried, and (4) greater therapeutic effectiveness with speedier response.

The technical advantages of administration of plasma in the hypertonic form as listed are obvious. Simplicity, safety, and speed attained by this technique are always important in the treatment of shock but are doubly so in military use. If plasma can be given in a shell hole, this is the way to do it. With a 50 or 100 c.c. glass syringe the hypertonic (four times concentrated) solution can be given as easily as 50 per cent glucose.

Technical advantages are significant only if therapeutic effectiveness is assured. Bond and Wright[9] first demonstrated that concentrated serum was effective in the treatment of shock in dogs. Best and Solandt[10] in a larger series also showed concentrated serum to be effective in the treatment of traumatic and posthemorrhagic shock in dogs, but later Magladery, Solandt, and Best[11] denied any advantage of concentrated over whole serum. Clinical reports by Ravdin, Stengel, and Prushankin,[12] Levinson, Rubovits, and Necheles,[13] and Strumia, Wagner, and Monaghan[14] have dealt with isolated cases of hypoproteinemia treated with concentrated serum or plasma, but its routine use as a superior therapeutic weapon in the treatment of shock was first reported in 1940.[3]

No large clinical series had been reported until the papers of Brown and Mollison,[15] and Hill, Muirhead, Ashworth, and Tigertt,[5] although preliminary summaries of comparatively large numbers of cases were given in our earlier papers. However, sublimation desiccation of plasma and its use in hypertonic form with emphasis on shock was practiced by the Baylor Plasma Service from its inception in August, 1939.

The advantages which we had at first assumed on theoretical grounds have since been brought out in extensive clinical trial. In Baylor Hospital alone concentrated plasma has been given to 276 patients. Of this number, 93 closely followed cases of shock are summarized and the evidence for hypertonic plasma presented in another paper.[16] The results have confirmed our previously stated thesis[3, 5] that the use of concentrated plasma is the ideal treatment of shock. The shock cycle of Moon[17] is quickly broken by a rapid increase of blood volume due to withdrawal of interstitial water. This constitutes a physiologic correction of the pathologic change of shock. Due to the speed of administration, this correction is effected in the shortest possible time. In addition, the extremely rapid increase in blood pressure of the average case strongly suggests a direct stimulation of the vascular system with reduction of vascular capacity.

In posthemorrhagic shock where red cells have not reached critical levels of oxygen carrying capacity, hypertonic plasma is superior to blood in its rapidity of action and instant availability. Where critical red cell levels have been reached, concentrated plasma is of utmost value in combating shock during the period of preparation and giving of blood. For a discussion of this important question reference should be made to an earlier paper.[6]

In answering the theoretical objection that dehydration would contraindicate the use of intravenous hypertonic solution, it is significant that we have found this type of therapy completely effective in the presence of all ordinary grades of dehydration seen in hospital practice. This is in accord with the well-known fact that interstitial water is easily mobilized and large in amount. According to Gamble,[18] this water makes up 15 per cent of the body weight and comprises a very effective reserve for the more important plasma volume. Of course severe dehydration approaching loss of 10 per cent of body weight would appear to be a definite contraindication for hypertonic plasma.

MASS PRODUCTION METHODS

The attainment of large volume production on our service has resulted not only from the adoption of new techniques but also from discarding two methods heretofore considered most essential[19]; namely, a closed type of centrifugation, and desiccation in original containers. These two requirements were abandoned because they constituted the chief obstacles to volume production. A partially open method such as described below has been proved perfectly safe in use with the observance of certain essential principles and checks.

First, it can be taken for granted that all precautions of careful technique and adequate bacterial culture control must be observed in any system. Second, conditions for bacterial growth must be completely eliminated following separation of plasma. If it must be stored before drying, it is kept frozen, but better yet is a balanced system where desiccation closely follows separation. In either event we have always employed Seitz bacterial filtration immediately prior to desiccation as an additional safeguard not only against bacteria but also possible pyrogenic substances. An important point in this connection is the use of filter-cel,* an infusorial earth preparation. This material is best introduced into some part of the system, such as the prefilter reservoir. It is sterilized with the equipment; when the plasma passes through the system, it is mixed with the filter-cel, and an even deposit forms over the surface of the Seitz disc. This coating greatly increases the capacity of the filter. After desiccation, bacterial growth is not supported and dryness is the greatest plasma safety factor. It can be handled in bulk with the same confidence as any other chemical or powder inert to bacterial growth, and it can be measured and placed in final containers with the same ease. It should, of course, be used immediately upon converting again into liquid form. Proper choice of unit sizes eliminates any need for brief storage as a liquid. Yet even here a safeguard is afforded by use in concentrated solution since this form is bacteriostatic to a large degree to all common contaminants.

It is obvious that the period of storage of liquid blood is the only break in the use of these principles. Where such preservation is essential to blood banking, safety can be attained by a completely closed collection system and a short period of storage. In our own service with 400 bleedings per month it has been feasible to keep blood only four to five days before separation of plasma. Of course, in large scale bleeding for national defense no storage would be necessary if the separator methods herein described were adopted for the immediate separation of plasma.

*A product of Johns-Manville Sales Corporation, New York, N. Y.

These principles have been worked out during the past two years in actual large scale use and have proved to be safe by the fact that no serious harm has resulted from a single dose, and a febrile reaction rate of 0.74 per cent has been attained in 694 administrations at Baylor Hospital. Furthermore, no reactions have been reported from extensive outside use.

The actual production methods, as shown in the American Medical Association Scientific Exhibit,[20] start with the bleeding technique. In our blood bank we have used a vacuum bottle collection technique. A rubber tube with two observation tubes and needles connected has been used. Vacuum is held during venipuncture by clamping a hemostat on the tube. Collapse of the tubing has in no way impeded withdrawal of blood. Our special mechanical shaker has been replaced by the simple expedient, now commonly employed, of inverting the vacuum bottle during collection. By this method the blood is drawn directly into the citrate solution, resulting in immediate mixing.

In bleeding for national defense we have suggested that defibrination might be preferable. Similar types could be bled into large chilled containers and defibrinated by a rotary paddle. The fibrin could be removed by a centrifugal strainer, and the bloods quickly pooled just before separation. Such methods have been standard in animal serum plants and should be considered seriously. Certainly the small hemoglobin content resulting from such methods must not be considered objectionable. We have given hemoglobin solution intravenously clinically up to 21 Gm. within fifteen minutes without reaction.

The second step of our methods is plasma separation. Two De Laval E-19 separators have been operated in cascade, the first unit separating and the second clarifying the plasma. With this smallest available motor-driven model, we have separated 25 liters of pooled blood in thirty minutes, a rate of 50 liters per hour. A true plasma yield of 50 per cent was obtained consistently. Of course, such capacities at high yields cannot be approached by conventional bottle centrifuges or, in fact, by any other methods. Furthermore, separators are available with capacities up to 1,000 liters per hour. Labor costs are reduced, and such large capacities with efficient yields make this method the obvious choice for mass separation. These conclusions are based on the separation of 778,280 c.c. of human blood by the continuous separator principle in four months. The pooling of whole blood prior to separation employed in our service since January, 1940, has many advantages. Typing is unnecessary, and large volumes are more easily handled. Moreover, agglutinins are completely removed, and the resultant agglutination of the corpuscles by forming larger particles aids in efficient separation and higher yields. Proportioning different types of blood or letting the pool stand as reported by Edwards, Kay, and Davie[21] is unnecessary, particularly with the large pools of 25 to 100 bottles which constitute part of our separation routine. We believe this removal of agglutinins resulting from pooling of bloods, as contrasted with the suppression resulting from pooling plasma of different types, is of the utmost importance. For example, we have never found it necessary to restrict the giving of whole blood after administration of large doses of our concentrated agglutinin-free plasma although Mahoney et al.[22] indicate that caution is necessary in the giving of blood following pooled plasma.

Disadvantages claimed for this method[23] have not been encountered during the eighteen months we have used it as a routine. First, contrary to these claims, high rates of hemolysis have not been found. By the method of Bing and Baker,[24] hemoglobin has varied between 20 and 75 mg. per 100 c.c. of unconcentrated plasma. The hemoglobin concentration has depended mainly on the age of the blood. We have discussed this question of hemoglobin elsewhere[6] and believe that it is of no significance whatever in the causation of reaction or other deleterious effects.[25] Indeed, the successful intravenous use of hemoglobin solution as a form of therapy reduces to an absurdity objections based on hemoglobin content.

Second, the method is partially open although we have redesigned covers and spouts to minimize this. Nevertheless, positive cultures were obtained on only three occasions, when the air was dusty during a two-day period from tearing down a nearby plaster wall. Since then, no contamination has been encountered, but, as an additional precaution, the air in our centrifuge room is electrostatically filtered.

Since the separation of plasma is immediately followed by Seitz filtration and desiccation, or freezing, filtration, and desiccation, bacterial contamination is no problem. We regard filtration of this type to be a safety factor of utmost importance as a final check against bacteria and pyrogenic substances. Since we have filtered the plasma from over 1,000,000 c.c. of blood, we cannot agree with Strumia and McGraw[8] that plasma filtration by this method is sufficiently inconvenient to be a valid objection to its use. Of course, precipitation of fibrin occurs, but it need not clog the filter if a filter of proper size is used and filter-cel added to the plasma to adsorb particulate matter, such as fat and fibrin. The decrease of fibrinogen content by filtration seems to be of no clinical importance.

After filtration, the plasma is delivered from the collector into the large processing ampoules by a bell-protected tip. Desiccation from the frozen state in bulk by the adtevac process[3] is then performed. This method utilizes the well-known fact that adsorbents, such as silica gel, have enormous capacities to take up water vapor. For efficient use in sublimation desiccation in vacuo, however, control of adsorbent vapor pressure and maintenance of capacity were required. These requirements, which had previously prevented the successful use of adsorption, were solved by the adtevac process. With maximum loads plasma moisture contents below the 1 per cent level set by Flosdorf and Mudd[1] were reached. With smaller loads moisture contents as low as 0.03 per cent as determined by the method of Flosdorf and Webster[26] were reached.

The chief characteristics of the process which make it ideal for large volume plasma desiccation are as follows:

1. It is not easily overloaded by initial rush of vapor in early phase.

2. Rapid spontaneous freezing eliminates expensive and troublesome prefreezing.

3. Temporary interruptions of electric current do not interfere with drying. Thawing and spoiling of plasma are avoided.

4. Extreme rapidity of freezing and positive maintenance of plasma in frozen state give high quality product with unusual solubility.

5. Reliability is assured by use of standard equipment such as vacuum pump and ordinary small refrigeration unit for machine parts.

6. There is economy of operation.

7. The process is well adapted to any size capacity required.

That desiccation from the frozen state need not be expensive has been amply demonstrated in the use of the adtevac process. Inefficient methods of separating and handling plasma on a large scale can easily increase labor cost more than the small power costs required for drying.

Transference of plasma from bulk-processing containers to final units is easily accomplished by a stainless metal dispenser that can be sterilized as a unit in the autoclave. With ampoules such as we have used, a closed system during transference can be had by using a compression joint and a flexible bellows or tube. A rotary-driven worm used in our first model granulated and forced the plasma through a small metal tip into the sterile vial standing on the pan of a torsion balance. The delivery tip was protected with a metal bell or hood. A later model uses a plunger type transfer mechanism. The plasma is considerably reduced in bulk during transference, and solubility is increased by the granulation. After sterile rubber caps have been applied, a high vacuum is drawn through a needle and the whole top sealed. Deterioration of vacuum is prevented by sealing the rubber cap with celloidin or suitable plastic solution. While we are constructing a dehumidified, air-conditioned, sterile room for our procedures, no difficulty has been encountered with these methods in an ordinary closed small room.

These bottles of plasma, together with enough pyrogen-free water to make the four times concentrate have been kept in our emergency room, in the obstetrical bags, and in the central laboratory. They have also been mailed to distant points in and out of Texas, and they have been used without trouble. This dry form invariably has been preferred to the frozen solution of concentrate which we had formerly used, due to greater availability and speed in preparing for use.

SUMMARY AND CONCLUSIONS

1. Mass production methods suitable for national defense needs and proved by extensive routine use are presented.

2. The complete safety of these methods is reflected in a febrile reaction rate of 0.74 per cent.

3. The use of serum separators and other partially open methods essential for volume output is safe under the conditions described.

4. The advantages of desiccated plasma prepared by the adtevac process are discussed.

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THE ASSAY OF HEPARIN*

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A NUMBER of different methods for assaying heparin have been described, some employing whole blood and some plasma. Some of these may be mentioned briefly. Jacques and Charles[7] recently reviewed three methods, namely; (1) a modification of the Howell method by Charles and Scott,[4] in which observations are made on the degree of clotting in samples of cat's blood containing varying amounts of the standard and unknown heparins; (2) a new thrombin assay, also a modification of the Howell method, in which a standardized thrombin is used to clot samples of oxalated beef blood containing varying amounts of the standard and unknown heparins, and (3) the Fischer and Schmitz method[6] in which the clotting time of chicken plasma on addition of thrombokinase is determined for varying concentrations of unknown and standard heparin. Chargaff[3] devised a method using chicken plasma, similar to that of Fischer and Schmitz but having the time constant and observing clot formation with varying amounts of heparin, following the addition of thrombokinase (the "muscle coagulin" of Fischer[5]).

In a recent communication, Schütz[9] describes a method in which rabbit's whole blood is added to varying concentrations of standard and unknown heparin solutions. The concentration of heparin in successive tubes is increased by 25 per cent. The comparison is made the following day, and the two racks containing the standard and unknown are moved along until there is a matching of the tubes. In the present method in which beef plasma is used, the concentration of each successive tube is increased by antilog 0.15 (about 40 per cent). With this interval, the clotting range may be covered with only 5 tubes. The standard and unknown tubes are graded according to a procedure to be described later, instead of matching directly as in Schütz and other methods.

The purpose of this paper is not to present a completely new method of assay but rather to discuss a technique of comparing the clots in the standard and unknown tubes employing a method already described by Reinert and Winterstein.[8] Only certain minor modifications have been made in their method in order that a statistical procedure may be applied to the results. Briefly, the method consists in observing the degree of clot formation at 37° C. in tubes containing heparinized citrated beef plasma to which calcium chloride has been added. The anticoagulating unit (a.c.u.) of Reinert and Winterstein is accordingly defined as that amount of heparin which inhibits for four hours the clotting of recalcified citrated beef plasma when kept at a temperature of 37° C. Five of these units equal one Best[1] or Charles and Scott unit, or simply, "Toronto Unit." (Reinert and Winterstein; also the author's evaluation.)

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The principles of evaluation described below can of course be applied to methods other than that of Reinert and Winterstein, but their method seems well suited to these principles, and with beef blood more readily available in larger quantities than blood obtained from the cat, rabbit, or chicken, more samples can be run simultaneously.

TABLE I

AMOUNT OF HEPARIN IN MICROGRAMS TO BE USED IN EACH TUBE

(Each increment = antilog 0.15)

TUBE NO.	SERIES 1	SERIES 2	SERIES 3	SERIES 4
1	0.71*	0.78	0.85	0.92
2	1.00	1.10	1.20	1.30
3	1.41	1.55	1.70	1.84
4	2.00	2.19	2.39	2.59
5	2.82	3.10	3.38	3.66

*In practice, the nearest twentieth of a microgram is used. This brings the volume measurements to the nearest hundredth of a milliliter.

TABLE II

VOLUMES OF REAGENTS USED IN SERIES 1

(Series 2, 3, and 4 are to be arranged similarly. The concentration of heparin is 5γ/ml.)

TUBE NO.	PLASMA (ml.)	AMOUNT OF HEPARIN		0.9% SALINE (ml.)	$CaCl_2$* (ml.)
		γ	ML.		
1	1	0.7	0.14	0.66	0.2
2	1	1.0	0.20	0.60	0.2
3	1	1.4	0.28	0.52	0.2
4	1	2.0	0.40	0.40	0.2
5	1	2.8	0.56	0.24	0.2

*See text for concentration of calcium.

PROCEDURE

Fresh beef blood is collected at a slaughterhouse (or preferably from the living animal by venipuncture) in two 1-liter bottles, each containing 50 ml. of 4 per cent sodium citrate solution. The blood is drawn into each bottle until the 500 ml. mark is reached. The mixed blood thus contains 0.4 per cent sodium citrate. On arriving at the laboratory, the blood is centrifuged for thirty minutes at 2,000 r.p.m. The supernatant plasma is placed in the reservoir of a 10 ml. Koch pipette. One milliliter portions are measured into sulfuric acid-dichromate-cleaned 12 by 100 mm. test tubes. To each tube are added appropriate amounts of heparin solution, 0.9 per cent saline, and calcium chloride solution so that the total volume is always exactly 2 ml. Five milliliter Koch pipettes graduated to 0.02 ml. are used for the latter solutions. The tubes are stoppered with snug-fitting corks paraffined only on the end (otherwise they tend to slip out) and inverted 2 or 3 times. They are then placed in a water bath at 37° C. and examined three hours later.

The standard and unknown heparin solutions are each made up in 0.9 per cent saline to contain 5 gammas of heparin per milliliter. In an assay there are 4 sets of 5 tubes each for each sample. The weights of heparin used in these 20 tubes are shown in Table I. Table II shows the arrangement of the 5 tubes of

the first series. For convenience, charts are used giving the actual burette readings for heparin and saline solutions for each series.

As indicated in Table II, the volume of calcium solution is 0.2 ml. Since different plasmas vary, the amount of calcium required varies and this amount must be determined on each new plasma before the assay is run. To do this, 6 tubes are set up, each containing 1 ml. of plasma, 2 gammas of standard heparin (0.4 ml.) and calcium chloride in the following amounts: 0.8, 1.0, 1.25, 1.5, 2.0, and 2.5 mg. respectively. The total volume in each case is 2.0 ml. The concentration of calcium chloride giving nearest to a 2+ clot (see below) after thirty minutes is selected and used for the assay. Occasionally, a longer time than thirty minutes is necessary. If the time is more than sixty minutes, the test must be repeated, possibly with higher calcium concentrations. From a stock solution of calcium chloride the assay solution is made so that the right amount of calcium is contained in 0.2 ml.

In conducting an assay, the 5 tubes for Series 1 are prepared for the standard heparin and for each unknown sample in succession. Series 2 is then similarly prepared and finally Series 3 and 4. In this way, any changes in the plasma due to standing have a minimum of influence on the results. As many as 6 unknowns can be conveniently run simultaneously with the standard.

READING THE TUBES

The reading of the tubes involves the use of a clotting scale based on the proportion of the total possible clot formed in each tube. Complete clotting (usually with marked retraction) is called 4+, and no clot formation is called 0. Intermediate values then are +, 2+, and 3+. On a percentage basis, + = 25 per cent, 2+ = 50 per cent, 3+ = 75 per cent, and 4+ = 100 per cent. The 2+ or 50 per cent clot formation has been adopted as the end point for comparing the standard and unknown heparins. This corresponds to the ED50 (50 per cent effective dose) of the usual dose-effect curve. All values are computed to this basis. It is apparent that not all partial clots will correspond exactly to +, 2+, or 3+, and that many clots will lie at intermediate values. Such intermediate values are designated by changes in the above symbols. For example, a clot which appears to be slightly heavier than 2+ is called 2+(+) or if slightly lighter, 2+(−). A 3+(−) clot would be slightly heavier than a 2+(+). Also, a clot that is not quite complete is called 4+(−), and a very small clot is 0(+). Thus there are 13 classifications, varying (theoretically) by 8⅓ per cent. It is not considered practical to attempt a finer classification under present conditions. Jacques and Charles[7] report: "At least four intermediate stages can be distinguished, and with practice, eight can be detected." This refers to whole blood, whereas, with plasma as in the present method, at least 11 intermediate stages can be ascertained with little difficulty.

One may ask, "Why resort to a clotting scale based on + values rather than one based directly on percentage, since the + values are ultimately converted into percentages?" The 3 principal + values are +, 2+, and 3+, corresponding to ¼, ½, and ¾ clots. It is easier for the eye to read, for example, "a

half clot plus a little more" [2+(+)] than "58⅓ per cent." In other words, the clot, after first rough appraisal by the eye, is graded more precisely by shifting the + value to one side or the other of the main classification. If a graded series of "standard clots" could be prepared and photographed, the task of reading would be simplified and a percentage scale could be employed. The difficulty arises from the variation of clot characteristics from plasma to plasma. Rate of shrinkage, volume of clot, opacity, flocculation, and even color are the variable factors that meet the eye. For example, a 4+ clot may be shrunken to a small volume or may show no retraction whatsoever at the time of reading.

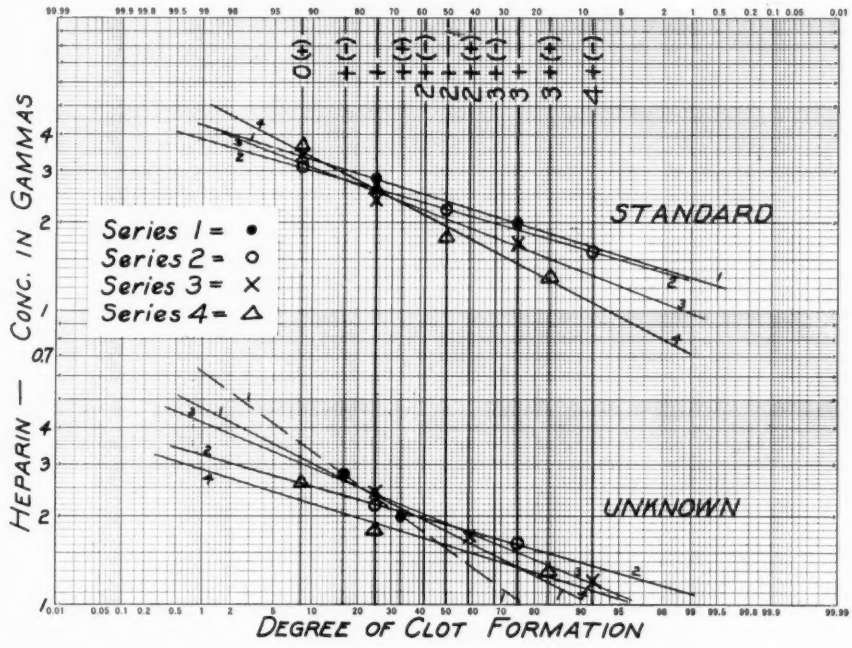

Fig. 1.—Curves showing the relation between the concentration and the degree of clotting on logarithmic probability paper. Solid lines: Best fitting straight lines for data on each set of tubes excepting Series 1 of the unknown. In this case, with only two points, the dashed line is the best fitting line, while the solid line is the more likely since it is more nearly parallel to the general slope.

It is obvious that the unaided inspection of a single tube may not accurately classify it. What might be identified as 2+ at one time may be considered 2+(−) at another. Therefore, the standard series is read first as carefully as possible, and the unknown tubes are then compared to these for grading. The tubes are in no sense paired as in the Schütz method, although frequently a tube from the unknown series may be observed to be identical with one of the standard tubes. Exact pairing facilitates the grading but is not necessarily the true end point.

The clotting process, as here observed, is not an "all or none" response and therefore is subject in a greater degree to the error of personal interpretation. In spite of this, it is believed that the end point here (defined as the formation of 50 per cent of the possible clot) can lead to a more accurate evaluation of anticoagulant activity than heretofore.

INTERPRETATION OF RESULTS AND CALCULATION OF POTENCY

After values have been assigned to each clot, an estimation of the concentration of heparin corresponding to a 2+ clot may be made by plotting the logarithms of the concentration of heparin against the probits of the graded clots or by plotting directly on logarithmic probability paper. The probits are determined by first converting the + values to percentages and the latter into probits by means of Bliss' table of probits.[2] The use of logarithmic probability paper has been the method of choice as it is more convenient and saves time. The data from a typical experiment are illustrated in Fig. 1. The heavily ruled ordinates correspond to the eleven + values (other than zero and 4+). They were drawn with India ink so as to remain as permanent reference lines while lightly penciled data could be quickly erased. There are usually 3 values that can be plotted (sometimes only 2 and sometimes 4), and the best fitting straight line is then drawn. (If the concentration intervals were less than antilog 0.15, more points would be available for plotting.) The "50 per cent dose" of heparin is indicated by the point at which this line crosses the 2+ ordinate. If only two points are obtained, it may happen that a line drawn through them does not have the usual slope. In such a case (see dashed line, Fig. 1) it should be drawn nearly parallel to the average slopes and between the points rather than through them, giving greater weight to the point nearest the 50 per cent or 2+ line. Slope as well as position must always be considered.

The calculation of potency may be made in a.c.u. (Reinert and Winterstein units) or in "Toronto Units." The following example is worked out in a.c.u. The standard contains 500 a.c.u./mg. (100 Toronto Units). If an estimation on one series shows the standard to have an equivalency of 2.35 gammas for a 2+ clot and the unknown 1.82 gammas, then the potency of the unknown is $500 \times 2.35 \div 1.82 = 645$ a.c.u/mg. This example, together with the other 3 series, is illustrated in Table III. For a complete assay the entire procedure is repeated on another day with a different sample of plasma. For the heparin assayed in Table III, the other four values were 600, 561, 622, and 614 a.c.u./mg. for the 4 series, respectively. The average for all 8 results was 595 ± 12 a.c.u./mg.

DISCUSSION

It may be asked if the relationship between the logarithms of the concentration of heparin and the probits corresponding to the percentage clot formation is actually linear or if it is a curved function. This cannot be answered with certainty at the moment, but in any event most of the data are randomly scattered about a straight line. When the majority of the data are not so scattered, the middle points are usually below the best fitting straight line and a curve drawn through them would have its convexity downward. This curving is slight in most cases and is not believed to be significant, but in any event the concurrent use of a standard neutralizes any such errors. Nevertheless, tests are under way to determine more accurately the exact nature of the dose-effect relationship.

A few points concerning the causes of error and variation in heparin assays may be briefly mentioned. There should be a minimum of time elapsing between

securing the blood and centrifuging it. Citrated beef plasma undergoes gradual changes on standing; therefore, a complete assay should be performed in the shortest space of time possible. In Table III it may be noted that the amount of heparin corresponding to a 2+ clot tends to diminish from the first to the fourth series. This apparent trend occurs as frequently in the opposite direction so is probably not significant unless it can be attributed to some changing characteristic of the plasma.

TABLE III

EXAMPLE OF THE ASSAY OF AN UNKNOWN HEPARIN AGAINST THE STANDARD

STANDARD HEPARIN			UNKNOWN HEPARIN		
AMOUNT OF HEPARIN (GAMMA)	READING OF TUBE	AMOUNT OF HEPARIN ESTIMATED FOR 2+ CLOT	READING OF TUBE	AMOUNT OF HEPARIN ESTIMATED FOR 2+ CLOT	POTENCY OF UNKNOWN (A.C.U./MG.)
0.7	4+		4+		
1.0	4+		4+		
1.4	4+	2.35	4+	1.82	645
2.0	3+		+(+)		
2.8	+		+(-)		
0.8	4+		4+		
1.1	4+		4+		
1.6	4+(-)	2.22	3+	1.86	596
2.2	2+		+		
3.1	0 (+)		0		
0.85	4+		4+		
1.2	4+		4+(-)		
1.7	3+	2.02	2+(+)	1.88	537
2.4	+		+		
3.4	0 (+)		0		
0.9	4+		4+		
1.3	3+(+)		3+(+)		
1.8	2+	1.90	+	1.62	587
2.6	+		0 (+)		
3.7	0 (+)		0		

When the tubes are being filled, especially with the plasma, the rate of delivery must be absolutely uniform and not too rapid. Since plasma is a moderately viscous liquid, a considerable amount adheres to the wall of the burette and drains slowly.

After the tubes are placed into the water bath, they should not be agitated. Any preliminary examination should be avoided or at least made with great care.

The volumes used are relatively small and a slight error in reading, even though no greater than the thickness of a graduation mark, can result in observable variation in the nature of the clot.

Cleanliness of the tubes is essential, the final cleaning being done with strong cleaning solution made of potassium dichromate and concentrated sulfuric acid. After thorough washing with tap and distilled water, the tubes are dried in an oven. If a brush is used in the preliminary cleaning before the acid, scratching of the glass should be avoided. The tubes should have as uniform a bore as possible.

Assays conducted in this laboratory have been on crude extracts as well as on the purified products. The crude extracts have shown potencies averaging about

400 a.c.u. per milligram, whereas the final product has averaged about 525 a.c.u. per milligram. This difference may not be the true difference in potencies since it has been pointed out by Jacques and Charles[7] that salts and other impurities cause variations in the clotting process to such a degree that the true anticoagulant value, due to heparin, may be considerably different than the observed value. Nevertheless, the results on many samples have been uniform within the bounds of reasonable consistency. Assays on 10 consecutive crudes and on finished products are given in Table IV.

TABLE IV

ASSAYS ON TEN SUCCESSIVE CRUDE AND PURIFIED HEPARIN SAMPLES

(Four tests made on each crude and 8 tests on each purified sample. Each group of 5 tubes comprises a "test.")

CRUDE HEPARIN		PURIFIED HEPARIN	
A.C.U./MG.	SM	A.C.U./MG.	SM
419	16	595	12
415	5	509	10
430	9	521	14
436	11	522	13
434	8	518	10
409	11	513	11
375	15	512	7
388	10	536	11
372	13	530	7
400	9	518	7
Mean 408		Mean 527	
SD = 23.5 units		SD = 25.1 units	

a.c.u., anticoagulating unit; Sm, standard error of the mean; SD, standard deviation.

There is probably no other biologic assay in which greater care must be exercised to obtain consistent results. The utmost in uniformity of manipulative procedure must be maintained at all times. This applies to the cleaning of the tubes and burettes as well as to exactness in measuring volumes, accuracy in preparing solutions, and the maintaining of constant time intervals for each step from start to finish. Surprisingly, however, the time of reading the tubes is of less importance than might be expected. It has frequently been observed that the majority of tubes show no observable change between two hours and four hours, the clotting curve apparently tending to level off after one or two hours. Further growth of clots after two hours, when they did occur, were rarely greater than one degree on the clotting scale. For this reason, the observation time of three hours was adopted as an average, and fifteen minutes one way or the other makes little difference. After twenty-four hours very few additional changes in clotting can be observed unless the tubes have been shaken.

SUMMARY

A technique has been described for determining the anticoagulant potency of heparin. The method consists in observing the degree of clot formation in recalcified citrated beef plasma to which varying amounts of heparin have been added. The degree of clotting is measured on a suitable clotting scale, after which the data in the form of the logarithms of the concentrations and the probits of the percentage clot formations are plotted to determine the "50 per

cent clotting concentration.'' The potency of an unknown preparation is estimated by comparing its 50 per cent clotting concentration with that of a standard. An assay is performed in several small series of tests so that a statistical evaluation may be obtained.

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A SIMPLE CLINICAL METHOD FOR DETERMINING SULFONAMIDES IN BLOOD*

A. Goth, M.D., Nashville, Tenn.

IN THE Bratton-Marshall method for determining sulfonamides in blood one has a very accurate and satisfactory method which is simple enough for all familiar with laboratory technique. This is not very time consuming if one has a well-equipped laboratory at hand, but it requires the making up of several quantitative solutions and cannot be carried out at the bedside. In order to make the determination of sulfonamides in the blood possible for clinicians in private practice and for those in small hospitals, a simple kit has been devised in which the reagents are put up in tablet form, with the exception of a small bottle of acetone.

The reagents are essentially the same as those in the Bratton-Marshall method with one major exception. Acetone is used for the precipitation of the blood proteins. After the blood has been shaken with acetone, the coagulated proteins settle out very rapidly, leaving a clear supernatant fluid, an aliquot of which can be used for the test. This avoids filtration and the necessity of carrying funnels and filters and saves considerable time. As the trichloracetic acid used by Bratton and Marshall is hygroscopic, it has been replaced by oxalic acid in the reagent tablet.

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As the determination of the combined sulfonamides is rarely necessary in practice, the test here described, which determines the free forms only, answers most of our clinical needs.*

APPARATUS

1. Pipette (1) 0.2 c.c. for measuring the blood.
2. Test tubes (2) 100 by 12 mm. marked at 1 c.c. and 3 c.c.
3. Comparator block with 6 color standards.

REAGENTS

1. Acetone.
2. Tablet No. 1 containing 30 mg. of oxalic acid.
3. Tablet No. 2 containing 1 mg. of sodium nitrite.
4. Tablet No. 3 containing 5 mg. of ammonium sulfamate.
5. Tablet No. 4 containing 1 mg. of N (1-naphthyl) ethylenediamine dihydrochloride.

In the preparation of the tablets, sucrose can be used to increase the bulk. The size of the tablets should not exceed 30 mg. to avoid unnecessary turbidity of the solutions.

TABLE I

AMOUNT OF PHENOLSULPHONPHTHALEIN (C.C.)	AMOUNT OF SULFANILAMDE INDICATED (MG. PER 100 C.C.)
0.09	2
0.18	4
0.27	6
0.42	8
0.60	10
0.77	12

PROCEDURE

1. Deliver 0.2 c.c. of blood into a test tube.
2. Add acetone to the 3 c.c. mark. Shake and let stand until clear (one to two minutes).
3. Transfer clear liquid into another test tube to the 1 c.c. mark.
4. Add water to the 3 c.c. mark and shake.
5. Add tablets No. 1 and No. 2, shake and let stand for three minutes.
6. Add tablet No. 3, shake and let stand for one minute.
7. Add tablet No. 4, shake, and after one minute compare with color standards.

In the kit mentioned above a color chart is used but where this is unavailable color standards may be made up as follows.

Dissolve 10 mg. of phenolsulfonphthalein in 250 c.c. of N/10 solution of sodium hydroxide. To each of six 100 by 12 mm. test tubes add 5 c.c. of N/10 sodium hydroxide solution and varying amounts of the phenolsulfonphthalein

*The preparation of reagent tablets by the physician is impractical. At our request, the A. S. Aloe Company, St. Louis, Mo., is supplying these, together with a complete kit for carrying out this test.

solution as indicated in Table I. The test tubes containing the color standards should be sealed and protected from direct sunlight. They should be renewed every six months.

The same procedure and color standards can be used for the determination of sulfanilamide derivatives. The amount of drug indicated by the standards should be multiplied by certain factors, when sulfanilamide derivatives are determined. These factors are 1.37 for sulfapyridine, 1.42 for sulfathiazole, and 1.45 for sulfadiazine.

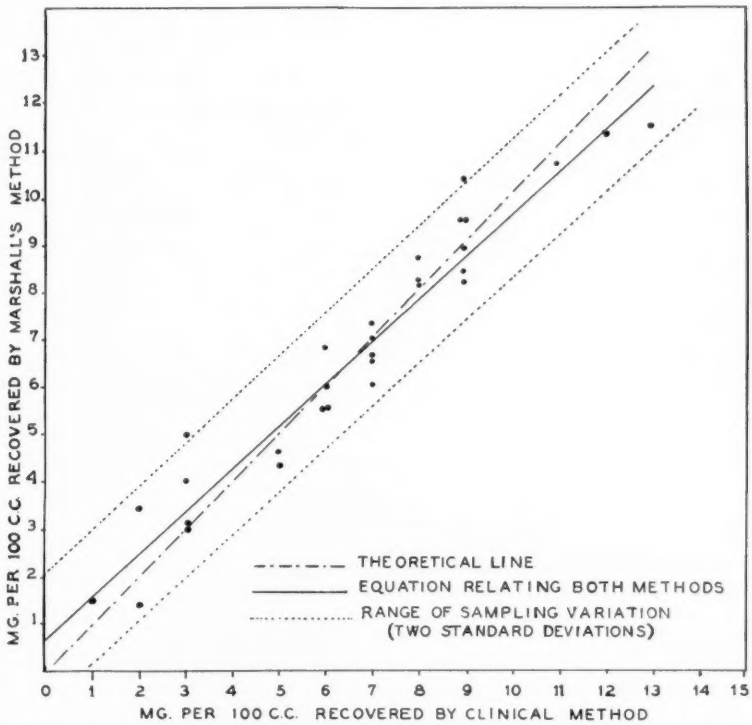
MG. PER 100 C.C. RECOVERED BY MARSHALL'S METHOD
MG. PER 100 C.C. RECOVERED BY CLINICAL METHOD
THEORETICAL LINE
EQUATION RELATING BOTH METHODS
RANGE OF SAMPLING VARIATION
(TWO STANDARD DEVIATIONS)

Fig. 1.—Recoveries of sulfanilamide in 30 blood samples. Comparison between the Bratton-Marshall method and the present clinical method.

In order to determine the accuracy of our method, 30 blood samples were analyzed with the Bratton-Marshall method and with our technique. As indicated in Fig. 1, our recoveries follow closely the ones obtained by the Bratton-Marshall method.

SUMMARY

A simple clinical method for determining free sulfanilamide and derivatives in the blood has been presented. All of the equipment required for the procedure can be placed in a small kit, and the test can be performed without laboratory facilities.

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PROTHROMBIN STUDIES USING RUSSELL VIPER VENOM*

III. Effect of Lecithinized Venom on Prothrombin Clotting Time

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THE use of Russell viper venom (Daboia venom) as a thromboplastin-like substance has been reported by many investigators.[1, 2] More recently this venom has been employed, instead of brain extract, as the thrombokinase in the determination of the prothrombin clotting time.[3, 4].

The coagulant action of Russell viper venom is potentiated by the addition of lecithin. Trevan and Macfarlane[5] found that the hemostatic action of Russell viper venom was accelerated by the addition of small quantities of tissue extract or lecithin.

Leathes and Mellanby[6] reported that lecithin from brain or egg yolk had the property of increasing the activity of the thrombokinase in Daboia venom.

Witts and Hobson[7] compared the prothrombin clotting times of 43 human beings using Russell viper venom (dilution 1:50,000) and lecithinized venom (adding 0.05 c.c. of a 10 per cent alcoholic solution of ovolecithin to each cubic centimeter of venom solution). The clotting times with lecithinized venom were shorter and less scattered than with venom alone. The usual time was eleven seconds with a suggested range of nine to thirteen seconds. With venom alone the clotting times were from two to nineteen seconds longer. In prothrombin deficiency, coagulation remains impaired despite the addition of lecithin to venom. They concluded that, if venom is used in the prothrombin clotting time test, it must be fortified by the addition of lecithin.

In a later paper Hobson and Witts[8] stated that Russell viper venom acts as a thrombokinase and accelerates the coagulation of blood or plasma in the presence of calcium and that the optimal concentration of the venom for use in the prothrombin clotting time test is 1:20,000. They conclude that, if Russell viper venom is used alone, the coagulation time may be affected by hemolysis, lipemia, the number of platelets in vivo, and, in certain circumstances, the speed and duration of centrifugalization, and that these inconsistencies are eliminated by the addition of lecithin to the venom.

Crosbie and Searborough[9] reported on coagulation studies with Russell viper venom alone and with venom plus a 10 per cent alcoholic solution of ovolecithin (B.P.C.†). Venom, in the absence of hemolysis and without lecithin,

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†The British Pharmaceutical Codex, 1934, p. 757.

is a more potent source of thromboplastin than is brain extract. When lecithinized venom was used, the coagulation times were shorter and the scatter was reduced.

Edsall[10] has reported that the coagulant action of Daboia venom appears to be exerted through an interaction with tissue extracts or with the cephalin contained in such extracts. Only a slight acceleration of coagulation was observed on employing 4 mg. of lecithin, comparable to that obtained with 0.002 mg. of cephalin. This may properly be ascribed to the unavoidable residual contamination of the lecithin with cephalin.

METHOD OF STUDY

Blood of patients in the hospital and of patients attending an outpatient clinic was obtained. Blood from patients, both with and without liver disease, was used. Four and one-half cubic centimeters of venous blood were drawn into a dry syringe and mixed with 10 mg. of potassium oxalate in a centrifuge tube. The blood was centrifuged at 1,500 r.p.m. for five minutes, and the oxalated plasma was drawn off. The test was performed within two hours after withdrawal of the blood, and, if hemolysis was present, the sample was discarded. The test was performed as follows: Two-tenths cubic centimeter of oxalated plasma was pipetted into a small test tube (75 by 10 mm.) and 0.2 c.c. of Russell viper venom,* 1:10,000 solution, was added. Calcium chloride solution (1.11 Gm. calcium chloride per 100 c.c.), 0.2 c.c., was then added, and the stop watch was started. The tube was agitated for ten to fifteen seconds in a water bath (37° C.), then removed and tilted until separate discrete fibrin particles could be seen. The first appearance of the fibrin particles was taken as the end point.

Prothrombin clotting time was determined on the undiluted plasma (100 per cent) and on 40 per cent plasma, diluted with physiologic saline solution.

The test was repeated, using the same plasma but with lecithinized venom. A 10 per cent alcoholic solution of lecithin† (egg) was used in the amount of 0.05 c.c. per cubic centimeter of venom. Venom of the same batch was used in both of the series of tests using venom and lecithinized venom. Each test was performed in triplicate, and the average time was taken for the result. The prothrombin clotting time in normal persons, by this method, for the 100 per cent plasma, is 20.76 seconds while that for the 40 per cent plasma is 33.10 seconds.[11]

RESULTS OF STUDY

The routine use of venom and lecithinized venom was carried out on 30 patients, and the resulting data on the 100 per cent plasma are outlined in Fig. 1 with the diagnosis of each patient. The complete data of the 100 per cent and 40 per cent plasma using venom and lecithinized venom are plotted in Fig. 2.

In the undiluted plasma studies the spread obtained with venom alone was from fifteen to thirty-four seconds, while with lecithinized venom it was from

*Supplied as 'Stypven' Russell viper venom by Burroughs Wellcome & Co., Inc., New York, N. Y.

†Lecithin (Merck).

thirteen to thirty seconds. In the 40 per cent plasma studies the spread obtained with venom alone was from twenty-seven to forty-seven seconds, while with lecithinized venom it was from 17.5 to 34 seconds.

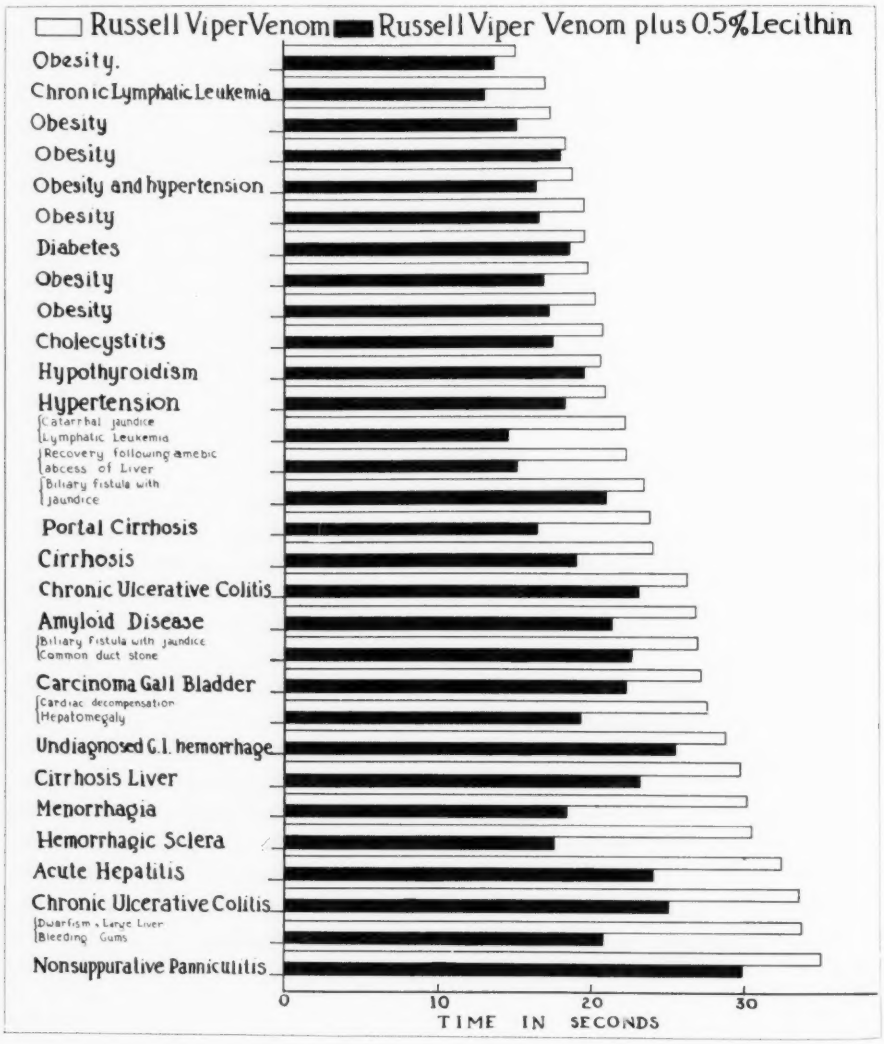

Fig. 1.—Bar graph showing comparison between prothrombin clotting times obtained with Russell viper venom alone and with Russell viper venom plus 0.5 per cent lecithin.

DISCUSSION

The addition of lecithin to Russell viper venom decreased the prothrombin clotting time in every instance (Figs. 1 and 2). An estimation of the extent by which lecithin shortened the clotting time was obtained as follows. The clotting times in which venom alone served as the thromboplastic substance were plotted against the corresponding times obtained with lecithinized venom and the equation for a straight line was calculated by the method of least squares. For undiluted plasma this was found to be $y = 5.74 + 0.554x$ where y represents the

expected clotting time for lecithinized venom and x represents the clotting time for venom alone.

Using this equation, one may obtain an idea of how effectively lecithin may be expected to reduce the clotting time. Substituting a value of twenty seconds for x one finds that the corresponding y value is 16.8 seconds, or, in other words, the presence of lecithin has reduced the expected clotting time by 16 per cent. If a greater clotting time, thirty-five seconds, is selected for x, the corresponding y value is 25.1 seconds and the reduction is 29 per cent. The percentage of reduction, therefore, is not constant but appears to be greater with prolonged clotting times. In the above discussion it must be borne in mind that the calculated values are the expected average values and the assumption was made that the data could be fitted by a straight line.

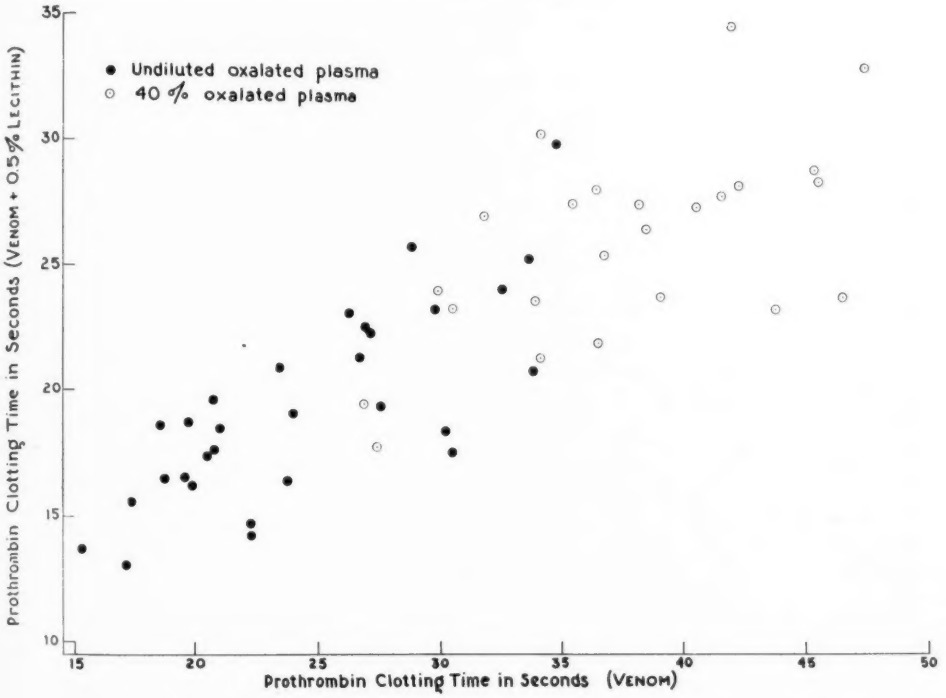

Fig. 2.—Comparison between prothrombin clotting time tests done with Russell viper venom alone and with venom plus 0.5 per cent lecithin on undiluted oxalated plasma and on 40 per cent oxalated plasma diluted with physiologic saline.

Certain prothrombin clotting times (Fig. 1) which would be classed as abnormal with venom alone and presumably by Quick's test[4] would fall into the normal range when lecithinized venom is used. For example, one patient with dwarfism, large liver, and bleeding gums (Fig. 1) had a prothrombin clotting time of 33.7 seconds with venom alone and 20.7 seconds with lecithinized venom. Another patient with hemorrhagic sclera (Fig. 1) had a prothrombin clotting time of 30.4 seconds with venom alone and 17.5 seconds with lecithinized venom. A third patient with menorrhagia (Fig. 1) had a prothrombin clotting time of 30.1 seconds with venom alone and 18.3 seconds with lecithinized venom. In each of these three patients the prothrombin clotting time with venom alone was

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prolonged, indicating prothrombin deficiency, while with lecithinized venom the prothrombin clotting time was in the range of presumably normal prothrombin times (with venom alone), i.e., 20.76 seconds ± 2.3 seconds.[11]

Lecithinized venom may fail to reveal moderate grades of prothrombin deficiency and, therefore, it is desirable to perform the prothrombin clotting time determination with venom alone. However, the use of lecithinized venom may prove to be a valuable adjunct, especially when more information has been secured as to the reasons for the difference between the prothrombin clotting times obtained with venom alone and with lecithinized venom.

SUMMARY

Prothrombin clotting time studies using a modified Quick's method employing Russell viper venom (Daboia venom) instead of brain extract have been carried out on 30 human beings.

Undiluted plasma (100 per cent) and 40 per cent plasma were used with venom alone and with venom which had been lecithinized by adding 0.05 c.c. of a 10 per cent alcoholic solution of lecithin (egg) to each cubic centimeter of venom.

The addition of lecithin to the venom accelerated the prothrombin clotting time in each plasma studied.

The percentage of reduction in the prothrombin clotting time is greater in plasma with prolonged clotting times than in plasma with rapid clotting times.

The prothrombin clotting time determination using Russell viper venom should be performed with the venom alone, and it is suggested that lecithinized venom be used as a supplementary procedure. The use of venom alone may reveal certain prothrombin deficiencies which are not evident when the venom is lecithinized. The use of lecithinized venom in some cases may prove to be a valuable adjunct, especially when more information has been secured as to the reasons for the difference between the prothrombin clotting times obtained with venom alone and with lecithinized venom.

We wish to express our appreciation for the cooperation of Dr. Louis Bauman in whose laboratory this work was done.

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DETERMINATION OF TRYPSIN IN DUODENAL CONTENTS WITH THE EVELYN COLORIMETER*

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WITH THE TECHNICAL ASSISTANCE OF HERMAN SIPLET, A.B.

DIFFERENT methods for the determination of trypsin in duodenal contents have been proposed. Some of them are based on the estimation of the nonprotein nitrogen in the protein-free filtrate after the digestion of casein with the duodenal contents (McClure[1]). Others determine the amount of amino acids in the filtrate by the Soerensen titration method (Leubner[2]). Hollander[3] and Lueders[4] measure the time in which a certain quantity of amino acids is formed in a gelatin solution. According to the law of Hedin, this time is inversely proportional to the concentration of the enzyme.

Anson and Mirsky[5] have developed a method for proteolytic enzyme determination in which they use a hemoglobin solution as a protein substrate and after digestion determine the amount of those substances which give a blue color with the phenol reagent of Folin and Ciocalteu.[6] The quantity of these substances formed during the incubation is a function of the enzyme concentration. Beazell, Schmidt, Ivy, and Monoghan[7] have adapted the pepsin method of Anson and Mirsky for the determination of pepsin in gastric juice. The trypsin method of Anson and Mirsky is likewise applicable for clinical purposes, and we shall describe the method as we have modified it for use with the Evelyn colorimeter.

The duodenal contents are centrifuged and diluted with physiologic saline solution 1:50. The assay of the enzyme should be performed as soon as possible after collection of the test material. In any case it should not be kept overnight. It has been our experience that during twenty-four hours about 30 per cent of the tryptic activity is lost even if the drainage material is kept in the refrigerator.

PREPARATION OF THE HEMOGLOBIN SUBSTRATE

The hemoglobin solution contains 2 per cent denatured hemoglobin and is buffered at a pH of 7.5. The solution is prepared according to the method of Anson.[8] Commercial products, such as Bactohemoglobin, give blank values which are too high, and therefore they should not be used.

INCUBATION

Five cubic centimeters of the hemoglobin solution are pipetted into test tubes. The tubes are stoppered with rubber stoppers and placed in a water bath, heated to 37.5° C., for about fifteen minutes to bring the hemoglobin solution to the right temperature. Then 1 c.c. of the diluted duodenal drainage is

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added in duplicate tubes. The contents of the tubes are mixed thoroughly by inverting several times. Then the tubes are replaced in the water bath. After fifteen minutes they are removed, and 10 c.c. of a 5 per cent solution of trichloracetic acid are added from another test tube into which it had been measured previously. The solutions are well mixed by pouring the contents back and forth from one tube to the other several times. The stoppers are replaced and the tubes turned upside down repeatedly.* To get a clear filtrate, the tubes must stand for about fifteen minutes. The solutions are then filtered through a Whatman filter No. 3. Three cubic centimeters of the filtrate are transferred into a colorimeter tube, and 20 c.c. of distilled water are added.

PREPARATION OF THE BLANKS

Blanks are prepared to determine the amount of preformed color-giving substances in the duodenal contents and the hemoglobin solution. To 5 c.c. of the hemoglobin solution 10 c.c. of 5 per cent trichloracetic acid are added. The tubes are stoppered and well shaken. Then 1 c.c. of the diluted duodenal juice is added, and the tube contents are thoroughly mixed. After fifteen minutes the solution is filtered. The filtrate of the blanks is treated the same way as the filtrate of the incubated samples.

PREPARATION OF THE STANDARDS

A stock solution of tyrosine is made by dissolving 15 mg. of tyrosine Pfanstiehl (which has been dried in vacuo over sulfuric acid) in 100 c.c. of water containing 0.5 per cent formalin. Before use the stock solution is diluted with distilled water 1:10. To a colorimeter tube are added 15 c.c. of distilled water, 5 c.c. of the diluted tyrosine solution, and 3 c.c. of 0.2 normal hydrochloric acid. The tyrosine stock solution can be kept for about two weeks in the refrigerator without change in strength.

DEVELOPMENT OF THE COLOR

To every colorimeter tube, including incubated samples, blanks, and standards, are added 1 c.c. of 3.85 normal sodium hydroxide and 1 c.c. of the phenol reagent (according to Folin and Ciocalteu).† The color reaches its maximum intensity in ten minutes and remains unchanged for a ten-minute period, during which the colorimeter readings should be made.

For adjusting the galvanometer scale to 100 the following "reading blank" is used. Into a colorimeter tube are pipetted 20 c.c. of water, 3 c.c. of 0.2 normal hydrochloric acid, 1 c.c. of 3.85 normal sodium hydroxide, and 1 c.c. of the phenol reagent.†

After the galvanometer scale has been adjusted to 100, the reading blank is replaced by the standard tube, and its color value is determined. It is advisable to read standards with every group of unknown samples as the phenol reagent is not absolutely stable and slight changes in it may occur. The Rubicon filter No. 660 is used. The calibration curve shows that this filter gives results in accordance with the logarithmic law of Lambert and Beer. After the color value of the standard has been determined, all incubated samples and blanks are read.

*In order to precipitate any of the material adhering to the stoppers.
†The tubes are stoppered and the contents are mixed by inversion.

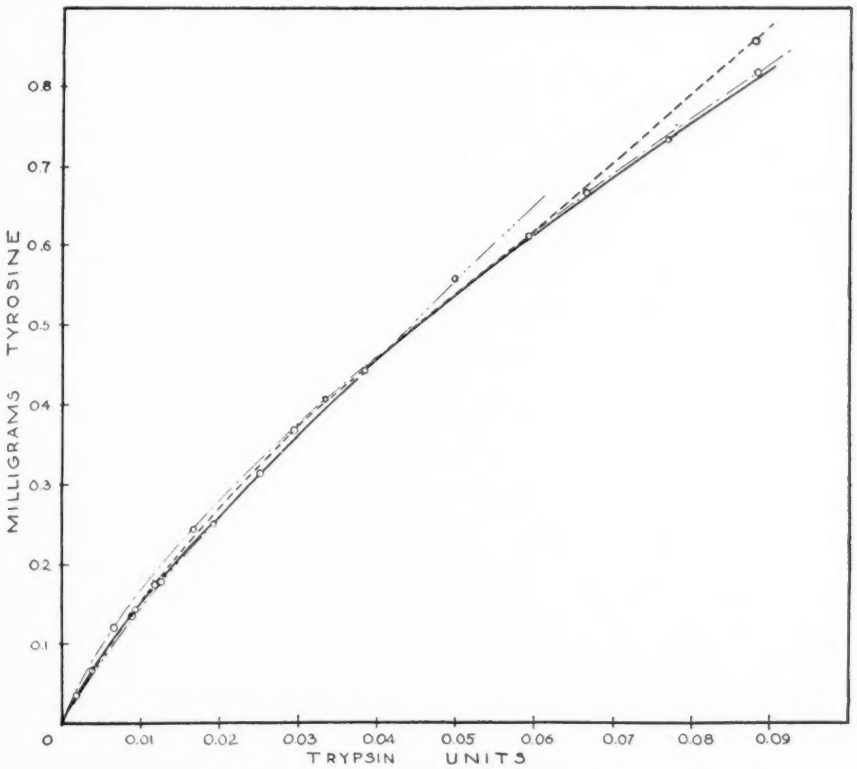

Fig. 1.—Four activity curves made from different duodenal contents.

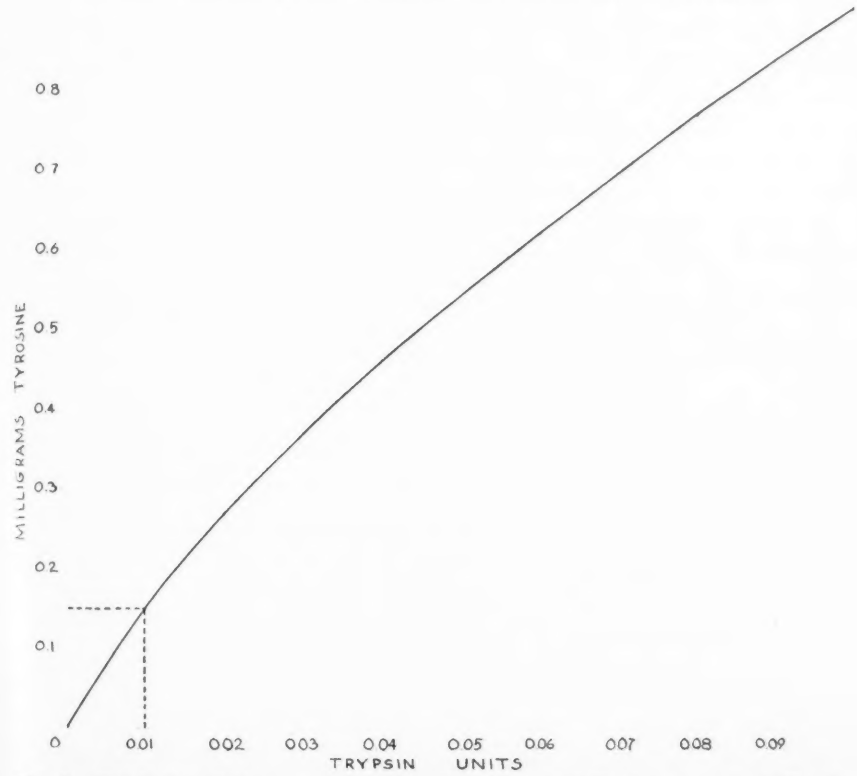

Fig. 2.—Standard activity curve represents the average of the four curves of Fig. 1.

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CALCULATIONS OF THE RESULTS*

$K = \frac{L}{0.075}$. K is the calibration constant; L is the photometric density of the standard; 0.075 is the tyrosine content of the standard in milligrams.

The quanity of tyrosine developed by 1 c.c. of the diluted sample is formed from the equation $C = \frac{L_1 - L_2}{K} \times \frac{16}{3}$. L_1 is the average photometric density of the duplicate incubated samples. L_2 is the photometric density of the blank. $\frac{16}{3}$ refers to the dilution. C = milligrams of tyrosine per cubic centimeter of the diluted duodenal material.

TRYPSIN UNITS

For our definition of a trypsin unit we have applied the definition which Beazell, Schmidt, Ivy, and Monoghan have given for a pepsin unit. Accordingly, one trypsin unit represents such tryptic activity which develops an equivalent of 1.0 mg. of tyrosine per minute under the conditions described. The results are expressed in trypsin units per 100 c.c. of duodenal material. For the conversion of the milligrams of tyrosine into trypsin units an activity curve was constructed in the following manner. Various dilutions of highly active duodenal contents were incubated with the hemoglobin solution; the developed quantities of tyrosine were plotted as ordinates, and the corresponding concentrations of the duodenal contents, as abscissae. Four such "activity curves" were then constructed from four different duodenal contents; in every curve the point in the abscissa corresponding to 0.15 mg. of tyrosine on the ordinate was marked to find the dilution of the duodenal content which corresponds to $\frac{1}{100}$ of a trypsin unit. From this the abscissa values were recalculated and expressed in trypsin units. The curves were thus reconstructed and brought into one coordinate system (Fig. 1). Fig. 2 represents the average curve of the four curves and can be used as a standard curve for converting the tyrosine values into trypsin units. Multiplication by 5,000 gives the trypsin units per 100 c.c. of the undiluted duodenal material.

The estimations performed in duplicates on 57 specimens show an average difference of 2.2 per cent between the duplicates.

CONCLUSION

A method has been described for the determination of tryptic activity in duodenal material by means of the Evelyn photocolorimeter.

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AN ADAPTER FOR MICROCENTRIFUGE SHIELDS*

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IN MICROANALYTICAL procedures the use of very small centrifuge tubes is not infrequently required. These tubes from 0.5 ml. to 3 ml. capacity must be suspended in correspondingly small holders called shields. We believe that the most commonly used type is the Cornell style, ½ inch outside diameter and about 2¼ inches long.† Even the smaller type centrifuges equipped with standard heads will not support these small shields but require a special head, costing several dollars. The larger floor type centrifuges which are most commonly used in the laboratory require an additional adapter to attach the microhead to the shaft.

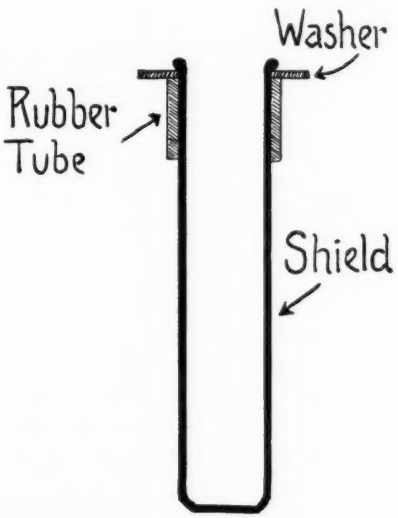

Fig. 1.

A very simple adapter is described which permits the use of the micro-shields with the ordinary head and shields for the 15 ml. centrifuge tubes.

*From the Department of Biochemistry, Marquette University School of Medicine, Milwaukee.

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†E. H. Sargent & Company, catalogue No. S17195.

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Fig. 1 illustrates the arrangement. An ordinary iron washer of 7/8 inch outside diameter is drilled out until the microshield can just be pushed through, a so-called sliding fit. After the washer has been pushed up until it touches the ring of the shield, it is held in place by a piece of heavy walled rubber tubing of 5/16 inch inside diameter and 3/32 inch wall. The pieces of tubing should be of exactly the same length for all of the shields used in order to equalize their weight. The rubber tube is slipped over a round piece of material, such as a dowel, is marked in 1/2 inch lengths, and is cut with a rolling motion with a very sharp knife. The washers must, of course, be of the same thickness and should be balanced before they are attached to the shields. Our shields with the adapter have the same weight within a range of 0.2 Gm.

SUMMARY

An easily made adapter for microcentrifuge shields is described. The changing of centrifuge heads is obviated.

THE DIRECT BIURET METHOD FOR THE DETERMINATION OF SERUM PROTEINS AS APPLIED TO PHOTOELECTRIC AND VISUAL COLORIMETRY*

GEORGE R. KINGSLEY, M.S., PHILADELPHIA, PA.

FURTHER investigation of the direct biuret method for the determination of serum proteins has led to several important refinements of the original procedure.[1] As modified, the method is more accurate, requires less time and fewer precautions than formerly, and is adaptable to visual as well as photoelectric colorimetry. An increase in concentration of the components of the biuret reagent permits accurate determination of serum protein up to 11 per cent. The use of a ready-mixed biuret reagent prevents precipitation of protein or cupric hydroxide. Such precipitation may occur if strong sodium hydroxide and copper sulfate solutions are improperly mixed in the presence of protein. The formation of turbidity, at times a complicating factor in the original method, is delayed by the addition of ether to the reaction mixture. Development of the biuret color under these controlled conditions is rapid, and readings may be made immediately after ether extraction and centrifugation. The improved direct method requires only ten minutes for the determination of serum total protein and twenty to twenty-five minutes for a protein fractionation.

REAGENTS

Biuret Reagent (TP) for Total Protein.—To 500 c.c. of 14 per cent sodium hydroxide ("carbonate free") in a pyrex bottle or flask, add 100 c.c. of 1 per cent copper sulfate ($CuSO_4.5H_2O$) solution and mix. Protect from dust and other contaminants. A rubber stopper may be used.

*From the Division of Biochemistry, Laboratories, Philadelphia General Hospital, Philadelphia).

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Biuret Reagent (ALB) for Albumin.—To 300 c.c. of 23 per cent sodium hydroxide ("carbonate free") in a pyrex bottle or flask, add 100 c.c. of 1 per cent copper sulfate solution and mix.

Ether.—U.S.P. grade is satisfactory.

Sodium Chloride.—0.9 per cent.

Sodium Sulfate.—23 per cent.

Standard Serum (for Calibration).—Obtain 5 to 10 c.c. of a single or mixed specimen of normal serum free from cells and hemolysis. Determine the protein concentration in duplicate samples by the macro- or micro-Kjeldahl method. Let C represent grams per cent total protein.

METHOD FOR PHOTOELECTRIC COLORIMETRY

Standardization.—Prepare stock standards in duplicate by measuring 1 c.c. portions of the analyzed serum into 10 c.c. volumetric flasks and diluting to the mark with 0.9 per cent sodium chloride. Mix. Prepare in duplicate a series of dilute protein standards by adding varying amounts of the stock standard and 0.9 per cent sodium chloride, as indicated in Table I, to 4 c.c. of the biuret

TABLE I

STOCK STANDARD (C.C.)	0.9 NaCl (C.C.)	PROTEIN EQUIVALENT (C) (GM./100 C.C.)
0.1	2.0	0.1C
0.2	1.9	0.2C
0.5	1.6	0.5C
0.7	1.4	0.7C
1.0	1.1	1.0C
1.2	0.9	1.2C
1.5	0.6	1.5C
Blank	2.1	0.0

reagent (ALB) as prepared for albumin determinations. Add 2 c.c. of ether to each tube, stopper, shake vigorously for approximately ten seconds, centrifuge for five minutes at about 2,500 r.p.m., and read in a photoelectric colorimeter. The readings of standard solutions are plotted against concentration of protein represented by each standard. From the graph so obtained colorimetric readings can be converted into concentration of protein. As an alternative, the concentration of protein in an unknown serum can be calculated[7] by substituting the readings of the unknown and standard sera as obtained in the biuret total protein determination and the protein concentration of the standard in the equation $X = (U - B)\frac{C}{R - B}$. X equals the grams of protein in 100 c.c. of the unknown serum; U, the reading of the unknown; B, the reading of the blank; C, the grams of protein in 100 c.c. of the standard serum; and R, the reading of the standard serum. $\frac{C}{R - B}$ need be determined only once (in duplicate) for each standard serum and will remain constant unless mechanical or other changes occur in the photoelectric colorimeter. The calibration should be verified periodically with fresh standard serum.

Total Protein.—To exactly 6 c.c. of biuret reagent (TP) in a photoelectric colorimeter tube add 0.1 c.c. of fresh serum (free from cells and hemolysis)

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with a micropipette after wiping the outside of the pipette. Rinse the pipette three or more times with the biuret reagent. Add 2 c.c. of ether, stopper, shake vigorously for approximately ten seconds, remove the stopper, and immediately centrifuge at about 2,500 r.p.m. for five minutes. Read in a photoelectric colorimeter within ten minutes after centrifuging. A green filter transmitting light between 500 and 570 mμ with maximum transmission at 540 mμ is satisfactory. The reading of the blank (Table I) is constant at 65-69 on the Klett-Summerson photoelectric colorimeter and needs to be determined only once for each new set of reagents.

If the serum is clear and contains only traces of hemoglobin and no more than normal concentrations of other pigments, fairly accurate results are obtained without ether extraction and centrifugation if readings are made five minutes after the development of the biuret color. When ether is not used, carefully mix the solutions by inverting; avoid shaking.

Albumin.[3]—Add 0.5 c.c. of serum to 7.5 c.c. of 23 per cent sodium sulfate in a test tube (18 by 120 mm.) and mix thoroughly by inverting. Add about 3 c.c. of ether and shake vigorously for twenty to thirty seconds. Cap or stopper the tube and centrifuge five to ten minutes at about 2,200 r.p.m. After centrifugation, slant the tube so that the tightly packed globulin precipitate separates from the walls of the tube. Insert a pipette through the ether layer preferably along the lower wall, withdraw 2 c.c. of the albumin solution, add to 4 c.c. of the biuret reagent (ALB) in a photoelectric colorimeter tube, and mix immediately. Then add 2 c.c. of ether and proceed as in the biuret method for total protein. Under certain conditions the use of ether may be omitted as stated above. To calculate the amount of albumin present, determine the protein equivalent of the reading by means of the graph or equation obtained by the standardization and multiply by 0.784.

Globulin.—Total protein minus albumin equals globulin.

METHOD FOR VISUAL COLORIMETRY

Standard I.—Add 1 c.c. of standard serum of known protein content and 1 c.c. of 0.9 per cent sodium chloride solution to 140 c.c. of biuret reagent (TP) and mix.

Standard II.—Add 2 c.c. of standard serum to 140 c.c. of biuret reagent (TP) and proceed as for Standard I.

Standard III.—Add 3 c.c. of standard serum to 139 c.c. of biuret reagent (TP). These standards will keep at least one month if stored in a pyrex bottle in a refrigerator.

For the determination of protein proceed as in the photoelectric method. Shake 6 c.c. of the standard biuret solutions with 2 c.c. of ether and centrifuge simultaneously with the unknowns. Remove sufficient colored solution from beneath the ether layer by means of a pipette and compare in a colorimeter against both a higher and a lower standard at room temperature. Use a green filter (maximum transmission at 540 mμ) in the colorimeter eyepiece. If the reading is within 10 to 15 per cent of the reading of one standard, the comparison with the other standard may be omitted.

Calculation.—$X = \dfrac{A\,\frac{r_a}{10} + B\,\frac{r_b}{10}}{2}$ where X is the protein concentration of the unknown in grams per 100 c.c.; A and B, the protein concentrations of lower and higher standards in grams per 100 c.c. of the standard serum; and r_a and r_b, the readings of lower and higher standards when the unknown is set on the left at 10 mm. A similar use of two standards has been described by Goudsmit[4] for the determination of creatinine by means of a photoelectric colorimeter.

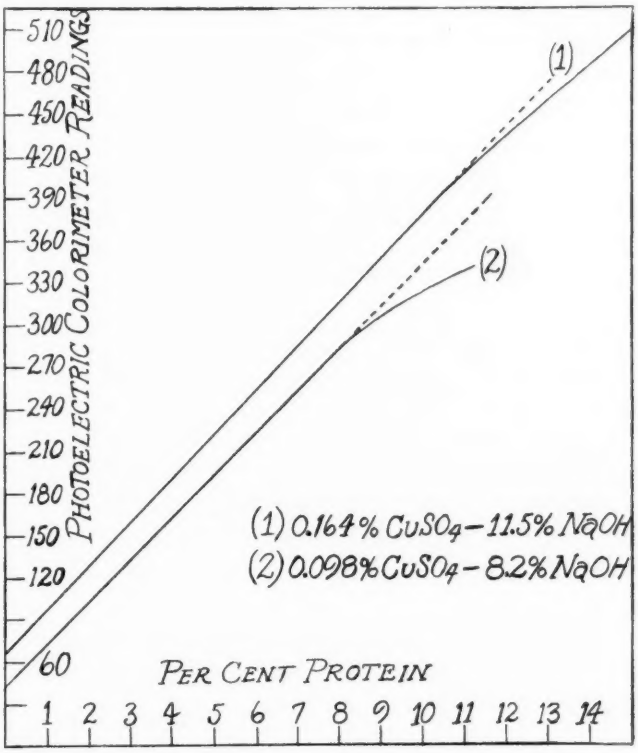

Fig. 1.—Biuret color intensity at increasing protein levels and the effect of different concentrations of copper sulfate and sodium hydroxide.

DISCUSSION

The biuret reaction mixture employed contains approximately 11.5 per cent sodium hydroxide and 0.16 per cent copper sulfate, concentrations that are considerably higher than those originally used.[1] When copper sulfate is increased to this strength, the ratio of protein concentration to biuret color produced is linear up to 11 per cent protein as shown in curve *1* (Fig. 1). Curve *2* represents the biuret color produced with the approximate concentration of reagents originally used. Increase in sodium hydroxide concentration also is necessary to avoid the precipitation of cupric hydroxide, especially on standing. Concentrations of sodium hydroxide as high as 13 per cent can be used, but 11.5 per cent was found to be sufficient for a stable reaction mixture. The presence of carbonate in the biuret reagent may cause precipitation with a

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decrease in color. For this reason sodium hydroxide solutions were prepared from clear 70 to 75 per cent solutions which had stood until sodium carbonate had separated.

Robinson and Hogden[2] stated that in the direct biuret method[1] the solutions were not perfectly clear, a straight line relationship did not continue above 8 per cent protein, and results comparable to their indirect method were obtained only by the most rigorous control of the time of reading; these objections do not apply to the modified procedure. Several of the time-consuming operations of the indirect method of Robinson and Hogden are avoided in the direct method since their procedure requires precipitation of protein with trichloracetic acid, the redissolving of protein precipitate with sodium hydroxide, and the removal of excess cupric hydroxide by centrifugation.

When ether is used, careful control of time of reading is unnecessary as solutions remain clear for about twenty minutes after centrifugation. On longer standing turbidity does develop, especially in some cholemic sera. However, if the solutions are again shaken with ether and centrifuged, clear solutions are obtained with unchanged protein values even though the solutions have stood for several hours.

The increased amount of copper employed results in a higher blank, and, at lower concentrations of protein, proportionately greater amounts of reagent uncombined with protein are present. The greater part of the light absorption of the excess biuret reagent is removed by the 540 mμ filter. Probably for this reason the 540 mμ filter has proved to be superior (in my experience) to the 560 mμ filter recommended by Robinson and Hogden.[2] However, if a visual colorimeter is used, it is necessary to employ a series of standards representing approximately 3, 6, and 9 per cent protein. The greater ease of operation and accuracy of the photoelectric colorimetric method makes its use preferable.

The 540 mμ Klett-Summerson filter absorbs all significant color due to the presence of bilirubin up to 25 mg. per cent. When 50 mg. per cent of bilirubin, a concentration rarely encountered in serum, was added to a serum containing 5.4 per cent protein, an apparent protein value of 5.5 per cent was obtained. Correction by means of a blank containing the added bilirubin and biuret reagent gave a value of 5.3 per cent; this indicates that the preparation of a blank to correct for the presence of bilirubin is unnecessary.

Slight hemolysis can be tolerated, but excessive hemolysis may give rise to errors. Attempts to correct for excessive hemolysis by means of a blank containing serum diluted with saline have been unsuccessful.

RESULTS

Excellent agreement is shown by the biuret and Kjeldahl methods when protein determinations are made on normal serum. The results of protein fractionation by the biuret and Kjeldahl methods of blood serums of 6 normal women and 9 normal men are presented in Table II. Kjeldahl determinations were made on 1 c.c. samples of serum. Boric acid was used in the distillation of the ammonia.[5] It is apparent that the accuracy of the biuret method does not differ significantly from that of the Kjeldahl method. In chronic liver disease dif-

ferences averaging 0.5 Gm. have been observed between the results of serum total protein determinations by biuret and Kjeldahl methods.[6] Further studies (to be published) also have shown small differences in some other diseases. It is debatable, of course, whether such differences are due to variation in total nitrogen, in biuret linkages, or both and consequently as to which method is giving results most nearly indicative of the actual concentration of serum protein.

TABLE II

DIFFERENCES BETWEEN BIURET AND KJELDAHL DETERMINATIONS IN NORMAL PERSONS*

		BIURET				DIFFERENCE (KJELDAHL MINUS BIURET)			
DATE	SEX	T.P. (GM. PER 100 C.C.)	ALB. (GM. PER 100 C.C.)	GLOB. (GM. PER 100 C.C.)	A/G	T.P. (GM. PER 100 C.C.)	ALB. (GM. PER 100 C.C.)	GLOB. (GM. PER 100 C.C.)	A/G
1/22/41	F	7.1	4.5	2.6	1.7	0.2	0.0	0.2	-0.1
1/22/41	F	7.2	5.4	1.8	3.0	0.0	0.1	-0.1	0.2
5/16/41	F	6.5	4.7	1.8	2.6	0.2	-0.1	0.3	-0.2
5/27/41	F	7.9	5.2	2.7	1.9	0.2	0.0	0.2	-0.1
5/20/41	F	7.0	5.4	1.6	3.4	0.1	0.0	0.1	-0.2
5/20/41	F	7.2	4.4	2.8	1.6	-0.1	0.0	-0.1	0.0
7/ 1/41	M	7.3	5.5	1.8	3.1	0.1	-0.1	0.2	-0.4
7/ 1/41	M	7.8	5.2	2.6	2.0	0.0	0.2	-0.2	0.3
7/ 1/41	M	8.3	5.9	2.4	2.5	0.0	0.0	0.0	0.0
7/ 8/41	M	7.3	5.6	1.7	3.3	0.1	0.1	0.0	0.1
7/ 8/41	M	7.6	5.8	1.8	3.2	0.0	0.0	0.0	0.0
7/ 8/41	M	7.0	5.5	1.5	3.7	-0.2	-0.1	-0.1	0.2
7/11/41	M	8.1	5.8	2.3	2.5	-0.2	-0.1	-0.1	0.1
1/22/41	M	7.2	5.1	2.1	2.4	0.1	0.0	0.1	-0.1
9/16/41	M	6.9	5.2	1.7	3.1	0.1	-0.1	0.2	-0.4
Mean		7.36	5.28	2.08	2.66	0.04	0.00	0.05	-0.04
Standard error		±0.035	±0.023	±0.041	±0.054				

*Three to four hours after breakfast.

SUMMARY

The direct biuret method for the determination of serum total protein and albumin has been modified and improved. Rapid and accurate determinations of serum proteins up to 11 Gm. per 100 c.c. in 0.1 c.c. samples of serum can be made by the new method. Application of the method to visual and photoelectric colorimetry is outlined.

I am indebted to Dr. John G. Reinhold, of the Division of Biochemistry, for suggestions and criticism.

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MEDICAL ILLUSTRATION

A SIMPLE METHOD FOR DETERMINATION OF EXPOSURE TIME IN PHOTOMICROGRAPHY*

HARRY A. TEITELBAUM, M.D., PH.D., AND JAMES C. PLAGGE, PH.D.
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TO THE nonprofessional photographer who does not use photographic equipment often enough to become expert in its manipulation, the simple application of mechanical devices that increase his efficiency is of great help. Photomicrography gives rise to problems which are particularly concerned with the amount of light that reaches the photographic plate. If the specimen being photographed varies in the intensity of the stain in different areas and also in thickness, as in supravitally stained tissue mounted *en masse*, it is difficult to determine by visual inspection the exposure time for different fields even in the same specimen. With sufficient experience one can estimate roughly the exposure time, but several photographs are often required to determine the correct time before satisfactory results are obtained. This frequently causes an unnecessary waste of time and materials.

The technique to be described uses standard equipment in a rather simple manner. A photoelectric cell (Fig. 1 *A*) was built into the wide end of a small camera having a fixed bellows (Fig. 1 *B*), the narrow end of which consisted of tubing (Fig. 1 *C*) about one-half inch long that fitted snugly into the microscope barrel in place of the ocular. The photoelectric cell was connected to an exposure meter (Fig. 2),† to record the intensity of light being transmitted through the microscope. Fig. 3 shows the complete equipment.

In using this equipment the field to be photographed is chosen and carefully focused. The microscope ocular is then removed, and the photoelectric cell is put in its place. A light reading is taken on the exposure meter. The photoelectric cell is replaced by the camera, and the focus is checked. The time of the first exposure is guessed. With the same setup additional shorter and longer exposures of the same specimen are made, the variations in time being figured in geometric progression. To economize on both photographic material and time, the trial exposure method,[1] in which several exposures are made on

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†This photoelectric exposure meter P. W. can be obtained from Pfaltz & Bauer, Inc., Empire State Building, New York.

one plate, may be used. In this way one eventually arrives at the correct exposure for a given reading on the exposure meter. This procedure is repeated for different meter readings.

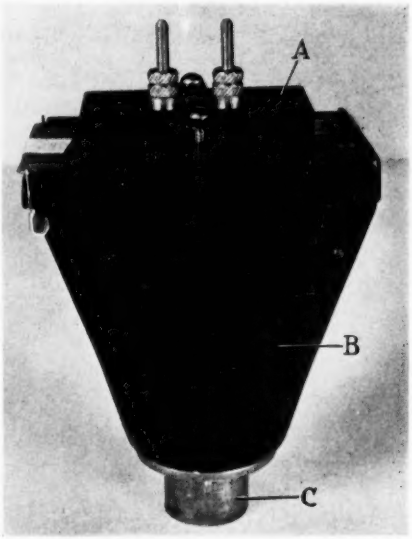

Fig. 1.—Photoelectric cell mounted on camera bellows. *A*, Photoelectric cell; *B*, camera; *C*, adapter for microscope tube.

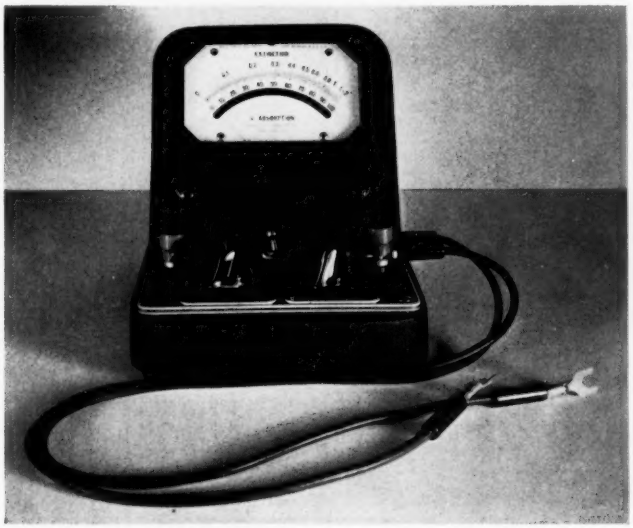

Fig. 2.—Exposure meter.

We used the foregoing method to establish a workable curve (Fig. 4) which would suit our particular purpose. After several successful photographs were made with different amounts of light, we plotted our points, using exposure meter units as the abscissa and the time of exposure as the ordinate. Then we drew a rough curve and joined these points. In order to add intermediate points to our curve and at the same time prove its validity, several additional points along its course were tested. Good negatives were obtained by using the meter

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readings and exposure times which fell on the curve. Negatives which were not made with the amount of light, and exposure time indicated by the curve were either overexposed or underexposed.

The curve obtained in Fig. 4 resulted when Wratten M plates and D-76c developer were used. Different types of plates would naturally give different curves.

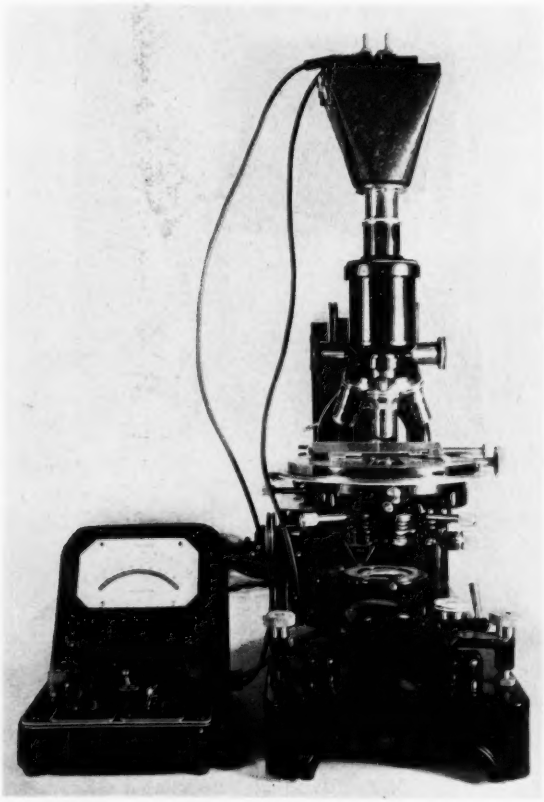

Fig. 3.—Complete equipment assembled with microscope.

Although the curve was constructed chiefly from low-power photomicrographs (×100 to ×200), it was tested for higher systems of lenses, including oil immersion, and found to be equally useful. Satisfactory pictures were obtained, regardless of specimen, thickness of section, stain, or magnification. The only requirement to be observed rigidly is that the exposure time be varied according to the exposure meter reading. The meter reading itself can be adjusted by changing the aperture of the diaphragm on the substage condenser, or in the light source if one is available there. This technique permits one to keep the intensity of light within the range of the curve that is most convenient for the exposure time units permitted by the mechanism of the camera shutter.

In Fig. 5 the application of the curve in Fig. 4 is illustrated. Extreme differences in light intensity and in magnification are shown in the four photographs. In *A* the amount of light employed was small, only 2 meter units, and the exposure time, as determined by the curve, was relatively long, one second.

In *B* the conditions were reversed, for the light meter reading was 55 and the exposure time was $\frac{1}{25}$ second. In *C* the magnification was low, ×100, the light intensity was 15 units, and exposure time was $\frac{3}{10}$ second; in *D* the magnification

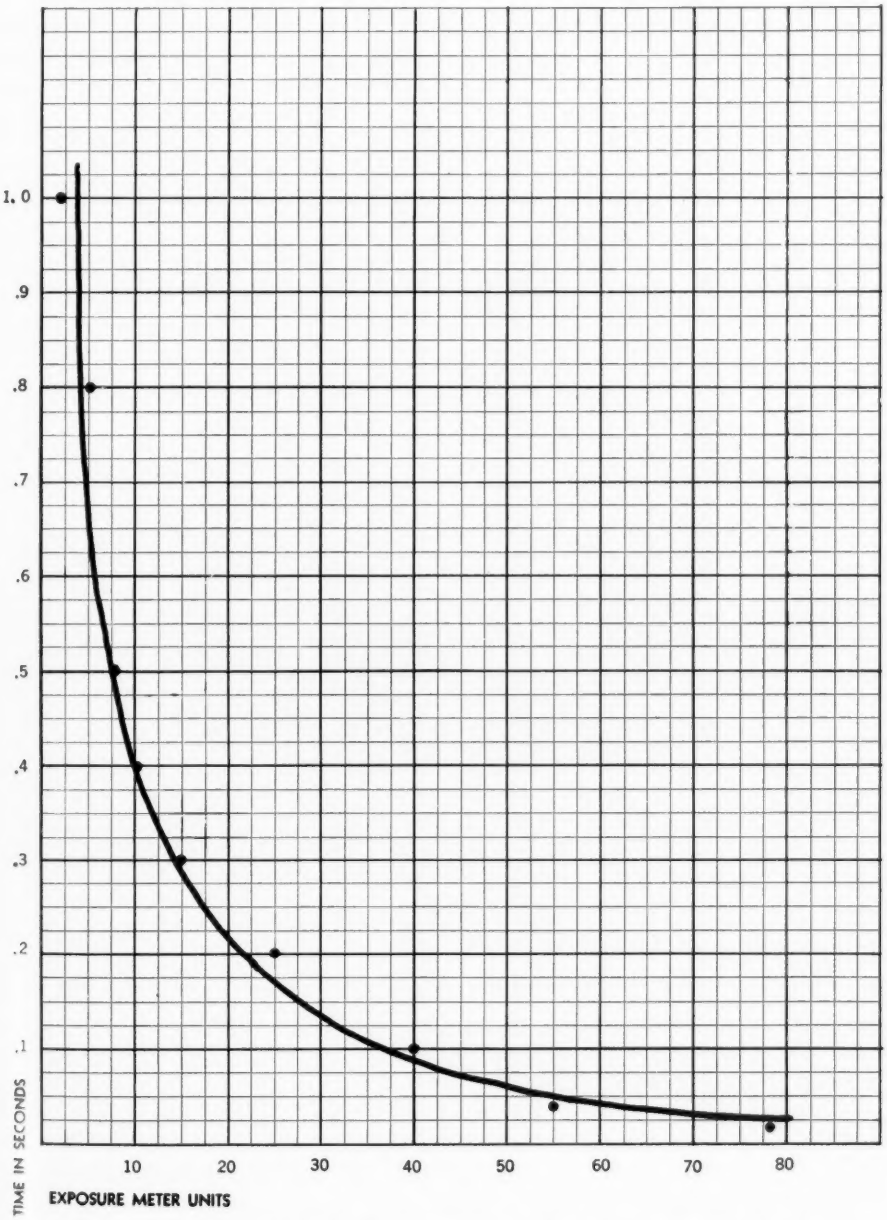

Fig. 4.—Graph showing relation of exposure meter units to exposure time.

was ×900, with the light intensity and exposure time the same as in *C*. The development and printing techniques were comparable in all instances. Slight variations in the depth of the final print, as in *A* and *B*, may be adjusted to suit one's particular purpose during the printing process.

When filters that exclude much of the available light are used, the equipment may not be sensitive enough to register the amount of light reaching the photoelectric cell. In such instances it was found advantageous to take exposure meter readings without the filters and to make trial exposures for determining the time with the filters in place. The correct exposure time was naturally much greater for any light meter reading than that recorded in Fig. 4, because the photographs were actually taken with much less light than that used for determining the exposure meter reading. A workable curve, similar to that in Fig. 4, was made for such low light intensities and long exposures.

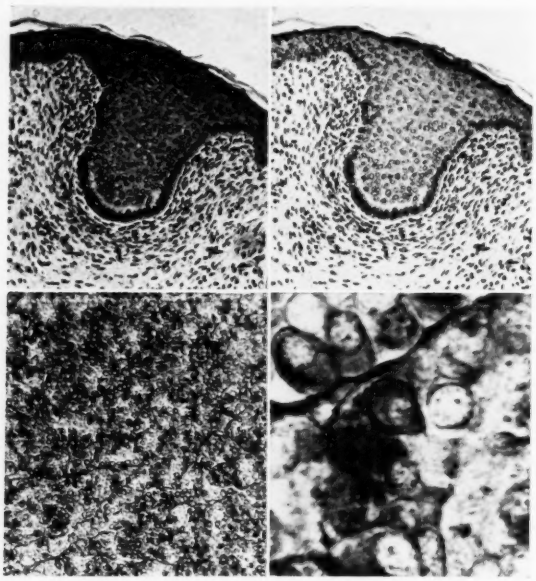

Fig. 5.—Sample photographs obtained by means of the graph illustrated in Fig. 4. The developing and printing processes were the same in each instance. *A*, Mammary gland anlage of treated pouch-young opossum. ×100. Exposure meter reading, 2 units; exposure time, 1 second. *B*, Same as in *A*, ×100. Exposure meter reading, 55 units; exposure time, 1/25 second. *C*, Anterior lobe of hypophysis of guinea pig. ×100. Exposure meter reading, 15 units; exposure time, 3/10 second. *D*, Same as *C*, ×900. Exposure meter reading, 15 units; exposure time, 3/10 second.

SUMMARY

A simple method for determining the exposure time in photomicrography by the use of a photoelectric cell and exposure meter is described.

We wish to express our appreciation to Professor Eduard Uhlenhuth for his interest and advice in working out this problem. We wish to give full credit to Doctors Raymond K. Thompson and Glenn H. Algire, who adapted the photoelectric cell as described in this paper.

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DEPARTMENT OF ABSTRACTS AND REVIEWS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

PERTUSSIS, a Note on the Cultivation of Hemophilus, Anderson P. M. M. J. Australia 2: 224, 1941

Flexible copper wire, gauge 19, was used in lengths of 15 cm., and on one end a tiny loop was made and dressed with a thin layer of cotton wool. These swabs were sterilized in tubes and were then ready for use. For the taking of the swab the child was seated on its mother's lap and the head was extended firmly against her shoulder. The manipulator bent over the child and passed the wire along the wider anterior nares into the post-nasal space. A side-to-side movement was then imparted to the swab, and the finger could feel the tip passing over the posterior pharyngeal wall. It was then quickly withdrawn, the end of the swab being depressed slightly and describing an arc. The wire was usually found to have taken on a curved shape.

Swabs were taken from children as young as 3 months old, and no difficulty was encountered; toddlers and older children occasionally coughed, but no failures occurred. The swabs were rubbed onto Bordet-Gengou medium containing 15 per cent of horse blood and incubated for four days, and a satisfactory proportion of positive cultures was obtained.

McCLURE-ALDRICH TEST. A Clinical Study and Evaluation, Lichtenburg, N. H. A. J. Dis. Child. 62: 743, 1941.

Thirty-three newborn infants given the McClure-Aldrich test on the first day of life and 50 infants tested after the "physiologic weight loss" was corrected had an average saline absorption time of 10.4 minutes.

Seventy normal newborn infants who were given no supplementary fluids and were tested at the time of greatest "physiologic weight loss" had an average saline absorption time of 26.3 minutes, two and a half times as long as that of the control groups.

Twelve normal newborn infants who had an average saline absorption time of 9.9 minutes on the first day of life showed an increase of absorption time to an average of 20.8 minutes on the third and fourth days, when the weight loss was maximum. The absorption time returned to an average level of 12.7 minutes on the day of discharge, when the weight loss and the state of hydration were, in part at least, corrected.

There was no difference between the absorption times of physiologic solution of sodium chloride and a solution of twice that concentration. Normal potassium chloride solution, however, was absorbed more than twice as quickly as physiologic solution of sodium chloride.

The McClure-Aldrich test is valuable in determining and following up the state of hydration of the body tissues. The absorption time depends, however, on the state of hydration at the site at which the test is performed and the mechanical ease of diffusibility of the injected saline solution into the intercellular spaces of the skin. It may be true that edema is a result of local or generalized toxicity and anoxemia and represents an attempt by the body to dilute the toxins. The result of the McClure-Aldrich test, however, may be regarded as indicative only of the extent and intensity of the edema, regardless of its cause. It may be a measure of tissue toxicity but only so far as, and when, the toxicity directly parallels the local edema.

NEPHROTIC SYNDROME, Acacia in the Treatment of, Goudsmit, A., Jr., Binger, M. W., and Power, M. H. Arch. Int. Med. 68: 701, 1941.

In 28 patients with the nephrotic syndrome who were treated with acacia the following observations were made:

1. The concentration of acacia in the serum resulting from the injection of 60 to 150 Gm. ranged between 1,000 and 2,500 mg. per 100 c.c.

2. The concentration of serum proteins usually diminished. As an average this amounted to 22 per cent of the original concentration. This related decrease could not be correlated with the amount of acacia administered, with the resultant concentration of acacia in the serum, or with the initial concentration of serum proteins. Increases in circulating plasma volume and concomitant dilution of its constituents appear to account adequately for the decrease of serum proteins.

3. The gross changes in colloid osmotic pressure subsequent to injections of solution of acacia are directly related to the absolute changes of the concentration of the serum proteins and of the concentration of acacia. If certain values are assigned for the "specific" osmotic pressures of serum proteins and of acacia, the changes of colloid osmotic pressure can be predicted fairly accurately. In approximately half of the patients the calculated changes were within 6 mm. of the observed values.

4. The colloid osmotic pressure of the serum may be increased, unchanged, or decreased after a course of injections of solution of acacia. An increase is most likely to occur when the initial colloid osmotic pressure is most subnormal.

5. These changes of colloid osmotic pressure, under the general regimen to which these patients were exposed, appear to be essentially unrelated to the degree of effectiveness of the treatment.

SULFANILAMIDE, A Simple Micro Test for, and Its Derivatives in Blood, Churg, J., and Lehr D. Am. J. M. Sc. 202: 589, 1941.

REAGENTS

A. 15 per cent aqueous solution of trichloracetic acid.

B. 2 per cent para-dimethylaminobenzaldehyde (p-BA) in 95 per cent ethyl alcohol. (If stored in a glass-stoppered brown bottle, the reagent keeps for at least a month. It is almost colorless if prepared from fresh p-BA. Crystalline substance which has turned strongly yellow due to prolonged standing is unsuitable for use.)

C. N/6 HCl (for determination of total sulfanilamide concentration).

EQUIPMENT

A. Test tubes, 10 mm. in diameter, calibrated at 1.5 c.c., 2 c.c., 3 c.c., and 4 c.c., with rubber stoppers.

B. Blood pipette, 0.1 c.c.

C. Small (1 inch) funnel and filter paper (Whatman No. 2, 4.25 cm.).

D. Graduated centrifuge tubes (for determination of total sulfanilamide concentration).

E. Comparator (block or slide) with blue glass filter, a set of permanent standards (Table I) and comparison tubes (10 mm. inside diameter) calibrated at 1 and 1.5 c.c.

TABLE I

COMPOSITION OF PERMANENT NONFADING STANDARDS

$K_2Cr_2O_7$ (C.C.)	$K_2Cr_2O_4$ (C.C.)	H_2O (C.C.)	CORRESPONDING CONCENTRATIONS OF SULFANILAMIDE IN BLOOD (MG. IN 100 C.C)
0.5	0.1	9.4	0
1.0	0.25	8.75	1
1.5	0.4	8.1	2
1.9	0.5	7.6	3
2.2	0.7	7.1	4
2.5	0.9	6.6	5
2.7	1.3	6.0	6
3.0	2.1	4.9	8
3.0	3.7	3.3	10
	0.5%		
3.0	5.0	2.0	15

PROCEDURE

A. Determination of Free Sulfanilamide

1. Place 1.5 c.c. of water in the calibrated test tube.

2. Add 0.1 c.c. of blood (drawn from finger or ear lobe) and rinse the blood pipette several times with the water in the tube. Shake the tube gently for a minute or two until the blood is completely hemolyzed.

3. To precipitate the proteins, add 15 per cent trichloracetic acid up to the 2 c.c. mark (approximately 8 drops). Close the tube with a rubber stopper and invert several times. This step may be performed immediately following 2, or within several hours.

4. After two minutes filter the mixture directly into the comparison tube up to the 1 c.c. mark. Instead of filtering, the mixture in the precipitation tube may be centrifuged for five minutes at high speed, and 1 c.c. of clear supernatant fluid transferred to the comparison tube by means of a capillary pipette. This is especially convenient if several tests are run simultaneously.

5. Add the p-BA up to the 1.5 c.c. mark, mix by inversion and compare with the standards in the comparator, either immediately or within two hours. In the latter case the comparison tube must be tightly stoppered.

The p-BA reagent should be shaken before use.

If the drug level in the blood is found to exceed 10 mg. per 100 c.c., accurate determinations are obtained by repeating the test with double dilution of the blood. For that purpose 0.1 c.c. of blood is hemolyzed in 3 c.c. of water and trichloracetic acid added up to the 4 c.c. mark (about 18 drops). Otherwise the procedure remains unchanged. The readings are multiplied by 2.

B. Determination of Total Sulfanilamide

The procedure as described above remains unchanged up to Step 4. Steps 4 and 5 are modified as follows:

4. Place 1 c.c. of the filtrate or centrifugate in a graduated centrifuge tube, add 1 c.c. of N/6 HCl, mix, and heat in a boiling water bath for one hour in order to hydrolyze the acetylated sulfanilamide. The mixture evaporates almost completely. Cool to room temperature and bring the volume up to 1 c.c. with water.

5. Add the p-BA reagent up to the 1.5 c.c. mark, mix by inversion, transfer into a comparison tube and compare with the standards. The readings are then multiplied by 1.15 to compensate for the decrease in color intensity due to the addition of hydrochloric acid.

C. Determination of Sulfanilamide Derivatives

With appropriate standards the test can be applied equally well to the determination of sulfapyridine, sulfathiazole, and other sulfanilamide derivatives. For clinical purposes, sufficiently accurate results can be obtained with these compounds by using the sulfanilamide standards for comparison, provided the readings are multiplied by the following factors:

	Free	Total
Sulfapyridine	1.5	1.7
Sulfathiazole	1.7	2.0

These factors take into account the higher molecular weight of sulfapyridine and sulfathiazole, the loss in precipitation due to the poor solubility of these compounds, and, in determination of total drug concentration, also the decrease in color intensity caused by the addition of hydrochloric acid.

T. N. T. Derivative in Urine, Improved Webster Test for, Ingham, J. Lancet 2: 554, 1941.

Place 50 c.c. of urine in a convenient sized beaker (400 c.c.) and add 50 c.c. of 20 per cent (by volume) sulfuric acid (1 part concentrated H_2SO_4 plus 4 parts H_2O). Mix.

Place beaker on tripod, supported by wire gauze, and heat over Bunsen flame till the liquid boils.

Boil for one minute; allow to cool. Cooling may be hastened by running cold water over outside of beaker.

After cooling, pour the mixture of urine and acid into a separatory funnel of 200 c.c. capacity and add 50 c.c. of ordinary (methylated) ether. Shake up well and then allow to settle for about fifteen minutes. The lower layer of urine and sulfuric acid is next run off by removing the top stopper and opening the lower tap.

The ethereal solution of acidified urine remaining in the separatory funnel is then washed twice with 1 per cent aqueous solution of sodium carbonate. This is done as follows. Add 20 to 25 c.c. 1 per cent sodium carbonate (aqueous), shake, and, after allowing to settle for five to ten minutes, run off the lower layer. Repeat. (If the person has been taking purgatives, such as rhubarb, senna, cascara, phenolphthalein, which yield anthraquinone derivatives, the sodium carbonate solution will show a reddish-violet color).

After this treatment with sodium carbonate the ethereal solution in the funnel is washed with water, as follows. Add 50 c.c. tap water, shake, allow to settle for fifteen minutes, and then run off the lower layer as before. (Occasionally a troublesome emulsion forms in the ethereal extract while washing with water but it is easily dispersed by adding a few drops of absolute alcohol and shaking gently.)

Now place 5 c.c. of the ethereal extract, so washed and prepared, in a test tube and quickly add, without shaking, 5 c.c. of a 5 per cent alcoholic solution of potassium hydroxide (5 gr. caustic potash dissolved in 100 c.c. of methylated spirit of absolute alcohol). A violet cloud indicates a positive result. The liquid in the test tube should be watched carefully, for the color develops quickly and just as quickly fades. The intensity of the color varies from the faintest tint to a deep violet according to the amount of TNT in the urine.

The unused ethereal extract remaining in the separatory funnel need not be thrown away but may be collected and later separated off from any TNT by distillation. It may then be used again in other tests.

For the routine everyday urine examination of workers in TNT factories the technique may be simplified as follows:

Apparatus Required.—Two ordinary test tubes (7 inches by ¾ inches); Bunsen burner or spirit lamp.

Reagents Required.—20 per cent (by volume) sulfuric acid; ordinary (methylated) ether; 5 per cent alcoholic solution of potassium hydroxide (freshly prepared).

Place about 10 c.c. of urine in one test tube; add about an equal quantity of the 20 per cent sulfuric acid; shake and bring to a boil; cool thoroughly under water tap.

When cold, add about 10 c.c. of the ether and shake well; allow to stand a few minutes until ether floats to the top. Pour some of the supernatant ether into the other test tube and to it add about 2 c.c. of the 5 per cent alcoholic potassium hydroxide solution. A dirty reddish purple color develops *at once* if TNT is present. The tint varies from faint to very deep according to the amount of TNT present and appears quickly and just as quickly fades. If no TNT is present, a light brown color only results and persists.

With this simplified technique the test is sensitive to 1 part in 10,000. It does not exclude anthraquinone derivatives (from the purgatives mentioned) which give a similar but pinker color which persists.

Erratum

On page 334 of the December issue of the JOURNAL in an article entitled "Age Incidence of Positive Tuberculin Reactions (Mantoux)" by Frances Pascher, M.D., and Marion B. Sulzberger, M.D. (with the statistical analysis by Alexander S. Wiener, M.D.), the formula appearing on the last line of the page should read:

$$\text{P.E.}_{\text{diff.}} = \sqrt{(\text{P.E.}_{p_1})^2 + (\text{P.E.}_{p_2})^2}$$

Also on page 335 of the same article the fourth line under Table III should read:

"Therefore, $p_2 - p_1 =$ 29.5 per cent and P.E. $(p_2 - p_1) = \sqrt{(0.06)^2 + (0.051)^2} = 0.081$, or 8.1 per cent."

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